

BACTERIOLOGICAL PROCEEDINGS-1972

G187 Physiology and Pathogenicity of Thermophilic Mutants of *Pseudomonas fluorescens*. Kerry F. Noon and B.T. DeCicco*. Catholic University of America, Washington, D.C.

Pseudomonas fluorescens (ATCC 13525) normally cannot grow at temperatures above 37°C. A series of heat tolerant spontaneous mutants of this organism were obtained by sequential selections at 45, 48, 50 and 52°C and were designated M45, M48, M50 and M52, respectively. The temperature range over which growth occurred, growth rates and thermal resistance were determined for each strain and demonstrated that while each organism was capable of growing over a temperature span of approximately 35 degrees C, the range shifted upward with each subsequent mutation. M52 had maximum, minimum, and optimum growth temperatures of 54, 20, and 49°C, respectively. The wild and mutant strains had almost identical nutritional, biochemical and morphologic properties. Antibiotic sensitivities of the mutant strains were similar to the wild type except that the mutants were more resistant to streptomycin and more sensitive to erythromycin, indicating that the original mutation to heat tolerance may have affected a ribosomal component. The LD₅₀ of the wild strain for male white mice was 1×10^9 organisms using the intraperitoneal route. LD₅₀ values for strains M45, M48, and M52 were 3×10^8 , 6×10^8 , and 1×10^{10} organisms, respectively, and correlated with each strain's growth capacity at 37°C.

BACTERIOLOGICAL PROCEEDINGS-1966

G87. A Method for the Determination of Low Frequency Transformations with *Hydrogenomonas facilis*. MICHAELEEN ZIOBRO and B. T. DE CICCIO. The Catholic University of America, Washington, D.C.

Transformation has been attempted in many gram-negative organism with only limited success. With *H. facilis*, the use of conventional transformation methods yielded neither significant nor reproducible results. Assuming that this lack of competence was due to an inadequate level of DNA penetration or incorporation, a system was devised which provided a long exposure period of cells to DNA and conditions completely selective for the survival of only transformed cells. Tubes of minimal broth containing wild-type *H. facilis* donor DNA and recipient cells of a multiple auxotroph were incubated and examined for periods up to 2 weeks. The occurrence of transformed cells or revertants was determined by visible growth. The test series yielded 80 to 100% positive tubes, while control tubes containing either DNA plus deoxyribonuclease or saline in place of DNA were 0 to 10% positive. A similar system was used for transfer of the streptomycin-resistant marker. Kinetic studies indicate that DNA penetration and incorporation occurs within 2 days after exposure. This sensitive method is now being used to determine whether long-term incubation will allow the quantitative measurement of competence levels in transformable as well as previously termed "nontransformable" strains.

RT22. Metabolism and Growth of *Hydrogenomonas facilis* and *Hydrogenomonas eutropha* under Autotrophic, Heterotrophic, and Simultaneous Conditions. PHILIP STUKUS and B. T. DECICCO. The Catholic University of America, Washington, D.C.

Since *H. facilis* and *H. eutropha* are facultative autotrophic hydrogen bacteria, they provided a means of comparing the utilization of inorganic and organic substances. In contrast to *H. facilis*, in which the hydrogen-oxidizing system is adaptive, this system in *H. eutropha* is constitutive. The hydrogenase activity of these organisms was studied to determine whether the oxidation of molecular hydrogen is affected by the presence of organic compounds. Growth of *H. eutropha* under "simultaneous" conditions, that is, when both autotrophic and heterotrophic substances are available, was measured and correlated with measurements of pH, hydrogenase activity, and the amount of organic substrate remaining. When cells of *H. eutropha* were grown in 0.4% glutamate in the presence of 70% H₂, 20% O₂, and 10% CO₂, an initial inhibition of growth was observed as compared to heterotrophic and other gas atmosphere controls. The inhibition of growth occurred between 1.5 and 3.5 hr postinoculation. After this interval, the inhibition was reversed, and growth of the organisms proceeded at a rate paralleling that of heterotrophically grown cells. A new method, employing auxotrophic mutants of *H. facilis* and *H. eutropha*, was used to demonstrate the simultaneous derivation of energy from inorganic and organic substrates. The results indicate that the organisms can derive a quantitatively significant portion of their energy from the oxidation of hydrogen, and this portion is influenced by the growth rate of the organisms, which is a function of the heterotrophic substrate employed.

P43. Amination-deficient Mutants of *Hydrogenomonas eutropha*. L. F. STRENKOSKI and B. T. DECICCO. Rensselaer Polytechnic Institute, Troy, N.Y., and Catholic University of America, Washington, D.C.

H. eutropha utilized inorganic nitrogen as sole nitrogen source for growth. Under such conditions, biosynthesis is dependent on reactions which incorporate ammonium nitrogen into organic molecules (amination). We isolated two mutant strains of *H. eutropha* which grew normally on most amino acids, but grew poorly or not at all under conditions requiring amination (nonamino acid carbon and energy source and inorganic nitrogen). Glutamate dehydrogenase, the only aminating enzyme present in wild-type cells, was found to be normal in mutant cells. Abnormal growth of mutant cells was not due to suboptimal utilization of nonamino acid substrates since mutant and wild-type cells oxidized these substrates at comparable rates. Additional studies with NH_4^+ ions as sole nitrogen source demonstrated that growth of both mutants was linear or approached linear values at rates dependent on extracellular pH and NH_4^+ concentration. Comparison of these results revealed that growth rates of mutant cells were proportional to the concentration of extracellular NH_3 . Wild-type cells were not dependent upon extracellular NH_3 since exponential growth rates did not vary with pH or NH_4^+ concentration. Both mutant strains were similar except for backmutation rates to the wild type. The results suggest that both mutants lack an NH_4^+ transport system and consequently must rely on NH_3 diffusion which does not support optimal amination rates.

GP113. Growth Requirements of *Hydrogenomonas eutropha* for Dense Cultures. R. REPASKE and C. A. AMBROSE, National Institutes of Health, Bethesda, Md., and Catholic University, Washington, D.C.

Autotrophically grown *Hydrogenomonas eutropha* is useful in the study of large-scale dense cultures, since toxic metabolic products often produced by heterotrophs do not accumulate and inhibit growth. *H. eutropha* has been grown in 23-liter batch cultures to 1.5×10^{11} viable cells per ml in less than 36 hr; the cell yields were 1,714 g (wet weight) and 497 g (dry weight). It was found that, previously, growth was limited by the diffusion of one of the gases; now maximum log growth (generation time, 1.7 hr) continued to an OD of 7. With supplements of Ni^{2+} and Fe^{2+} , that rate was maintained to OD 14. Thereafter, another limiting mineral reduced the rate by one-third, but growth continued to be logarithmic to OD 50. Lag periods with inocula of different ages were eliminated by adjusting the initial $p\text{CO}_2$: log phase cells required 1.5% CO_2 , late log cells required 4% CO_2 , and inocula in the stationary phase grew immediately if the $p\text{CO}_2$ were 8%. Under appropriate conditions, logarithmic growth of *H. eutropha* in 23-liter batch cultures was maintained from the time of inoculation (OD_{660} of 0.050) to an OD of 50.

Autotrophic and Heterotrophic Metabolism of *Hydrogenomonas*: Regulation of Autotrophic Growth by Organic Substrates

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The effects of a number of organic substrates on the autotrophic metabolism of *Hydrogenomonas eutropha* were examined. Dual substrate (mixotrophic) cultivation in the presence of hydrogen plus either fructose or alanine allowed autotrophic growth to begin immediately after the exhaustion of the organic substrate. On the other hand, the presence of acetate, pyruvate, or glutamate caused a lengthy lag to occur before autotrophic growth commenced. With acetate or pyruvate this lag (plateau) in the dicyclic growth curve was due to the repression of ribulose diphosphate carboxylase (RDPC) synthesis during mixotrophic growth. During heterotrophic growth with glutamate, RDPC was partially repressed; however, during mixotrophic growth, RDPC activity was high. Thus the delay of autotrophic growth was not due to a repression of RDPC by glutamate. The data suggest that glutamate interferes with autotrophic metabolism by repressing the incorporation of inorganic nitrogen. The repression of these vital autotrophic functions by acetate, pyruvate, and glutamate occurred both in the presence and absence of hydrogen, i.e., during both heterotrophic and mixotrophic cultivation. The derepression of the affected systems during the plateau phase of the dicyclic growth curves was demonstrated. Carbon dioxide assimilation by whole cells agreed well with the RDPC activity of extracts from cells grown under similar conditions.

A number of organic compounds, utilizable as carbon and energy sources for heterotrophic growth, can affect the autotrophic metabolism of hydrogen bacteria. In cells grown heterotrophically, these effects were observed as decreased levels of the two vital autotrophic functions, the hydrogen-oxidizing and carbon dioxide-fixing systems. Among the members of the genus *Hydrogenomonas*, *H. flava* (9), *H. facilis* (13, 20, 23), *H. ruhlandii* (16), and *Hydrogenomonas* H 1 (2) possess inducible or repressible hydrogenases. Only *H. eutropha* seems to lack this regulatory mechanism (Bovell, Ph.D. Thesis, University of California, Davis, 1957; Stukus, M.S. Thesis, The Catholic University of America, Washington, D.C., 1966).

The carbon dioxide-fixing system plays perhaps an even more critical role in the control of autotrophic metabolism. In *Hydrogenomonas*, as in other autotrophic bacteria, considerable evidence implicates ribulose diphosphate carboxylase (RDPC, EC 4.1.1.39) as the primary

catalyst for the assimilation of carbon dioxide during autotrophic growth (1, 3, 7, 8, 14, 17, 19, 21). The activity of this enzyme in a number of facultative autotrophs after heterotrophic cultivation was reported to vary from 0 to 100% of the autotrophic level, depending upon the growth conditions employed. Growth on pyruvate (8), acetate, or malate (5) apparently represses RDPC in *Chromatium*. A similar repression by acetate was observed with *Micrococcus denitrificans* (10) and *H. facilis* (15), suggesting that RDPC plays a critical role in the control of CO₂ assimilation during heterotrophic growth. *H. ruhlandii*, when grown on lactate, possessed only 2% of the autotrophic level of RDPC (22), whereas *H. eutropha* contained much higher levels (18). Growth on fructose produced decreasing levels of RDPC with *Hydrogenomonas* H 16 (6) but not with *H. facilis* (15). More recently, other environmental conditions, including the degree of aeration, were implicated in the maintenance of high RDPC activity during heterotrophic growth on fructose (11).

We previously described the dicyclic growth

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patterns which occurred when *H. eutropha* was grown under dual substrate (mixotrophic) conditions (4). When the organic substrate allowed simultaneous autotrophic and heterotrophic metabolism during the initial growth phase, as evidenced by increased cell yields, a rapid change-over to exclusively autotrophic growth occurred at the point of organic substrate exhaustion. With three of the tested organic substrates, glutamate, acetate, and pyruvate, the initial growth phase seemed to represent heterotrophic rather than simultaneous growth, and with these substrates a lengthy changeover period of 5 to 8 hr preceded the initiation of autotrophic growth. It was suggested that both the lack of simultaneous growth and the lag (plateau) before autotrophic growth commenced were due to a repression of some autotrophic function by these three substrates. Since hydrogenase is constitutive in *H. eutropha*, and since decreased levels of RDPC were reported in some facultative autotrophs after heterotrophic growth on glutamate, acetate, and pyruvate, it seemed likely that these substrates may be repressing RDPC in *H. eutropha*, both in the presence and absence of the autotrophic atmosphere.

The present investigation was undertaken to determine whether the growth patterns observed under dual substrate conditions could be correlated with RDPC activity. In addition, $^{14}\text{CO}_2$ assimilation by cells grown under various conditions was compared to their RDPC activities to confirm that this enzyme is the major catalyst for autotrophic carbon dioxide-fixation in *H. eutropha*.

MATERIALS AND METHODS

Special chemicals. 3-Phosphoglyceric acid kinase, 3-phosphoglyceraldehyde dehydrogenase, and adenosine triphosphate (ATP) were obtained from Calbiochem, Inc., Los Angeles, Calif.; 2,5-diphenyloxazole (PPO), dimethyl 1,4-bis-2(5-phenyloxazolyl)-benzene (POPOP), and Triton X-100 were from Packard Instrument Co., Downers Grove, Ill.; $\text{NaH}^{14}\text{CO}_3$ was from Schwarz BioResearch Inc., Orangeburg, N.Y.; dibarium ribulose-1,5-diphosphate and reduced nicotinamide adenine dinucleotide (NADH) were obtained from Sigma Chemical Co., St. Louis, Mo.; Nessler's reagent was from Arthur H. Thomas Co., Philadelphia, Pa.

Media and growth conditions. Autotrophic and dual substrate cultivation of *H. eutropha* were as previously described (4). For heterotrophic growth, organic substrates which had been autoclaved separately in distilled water were added to the specified concentrations, and incubation was under air. Because of the influence of shaker speed on RDPC activity noted by Kuehn & McFadden (11), RDPC was determined in extracts from cultures which had been shaken at both slow (130 gyrations/min) and

rapid (325 gyrations/min) speeds. Growth was monitored by following the increase in optical density (OD) at 540 nm by using a Spectronic-20 colorimeter (Bausch and Lomb, Inc., Rochester, N.Y.). An OD of 1.0 corresponds to a cellular dry weight of 0.32 mg/ml, and this relationship is linear throughout exponential growth.

Cell-free extracts. Cells were grown under the specified conditions, harvested by centrifugation at 2°C, and suspended in 0.05 M tris (hydroxymethyl) aminomethane (Tris)-hydrochloride buffer (pH 7.9) containing 12 mM of β -mercaptoethanol, 50 mM NaHCO_3 , and 1.5 mM ethylenediaminetetraacetic acid. The cell suspensions were disrupted by sonic treatment for 2 min in an ice bath by using a Biosonik II sonifier (Bronwill Scientific Co., Rochester, N.Y.) at maximum power. The disrupted suspensions were centrifuged at $20,000 \times g$ for 20 min at 2°C. The supernatant fractions were dialyzed overnight at 4°C against 4 liters of the same buffer solution in which the cells had been suspended. These dialyzed extracts were used in the RDPC assay.

RDPC assay. The method employed was a modification of that described by Hurlbert and Lascelles (8). The assay consisted of two parts. The first part involved the formation of 3-phosphoglyceric acid (3-PGA); the second part was the spectrophotometric determination of the amount of 3-PGA formed in part one measured by the oxidation of NADH. The reaction mixture for part one contained 12 μ moles of MgSO_4 , 12 μ moles of reduced glutathione, 80 μ moles of NaHCO_3 , 1 μ mole of ribulose diphosphate, 100 μ moles of Tris-hydrochloride buffer (pH 7.9), and the amount of cell extract containing 1.0 mg of protein, in a final volume of 2.0 ml. The mixture was incubated at 37°C for 5 min and then placed in a boiling water bath for 3 min to stop the reaction. After cooling, the mixture was clarified by filtration through a Swinnex 13 membrane filter unit (Millipore Corp., Bedford, Mass.) with a pore size of 0.45 μ m. The filtered material was used in the spectrophotometric assay which was performed by using a Hitachi Perkin-Elmer 139 spectrophotometer equipped with a Coleman-Hitachi 165 recorder. For the assay, the cuvettes contained 17 μ moles of MgSO_4 , 20 μ moles of L-cysteine, 2 μ moles of ATP, 0.4 μ moles of NADH, 150 μ moles of Tris-hydrochloride buffer (pH 7.9), 210 μ g of 3-phosphoglyceraldehyde dehydrogenase, and 1.0 ml of filtered material, in a final volume of 3.0 ml. The reaction was started by the addition of 2.2 μ g of 3-PGA kinase. The oxidation of NADH was measured as the decline in OD at 340 nm until there was no further decrease, and the amount of 3-PGA in the sample was calculated from the amount of NADH oxidized. A unit of enzyme activity is defined as the amount catalyzing the formation of 1 nmole of 3-PGA per min which corresponds to a decrease in OD of 0.0045. Specific activity is defined as units per milligram of protein. Protein was determined by the biuret method.

Fixation of labeled bicarbonate. Cells were grown under the specified conditions, harvested at the desired growth phase by centrifugation at 2°C, and resuspended to 4.0 OD in 0.04 M potassium phosphate

buffer (pH 7.2). Incorporation of $^{14}\text{CO}_2$ was performed by using double side-arm Warburg flasks. The main compartment of the flask contained 1.0 ml of the cell suspension and 1.5 ml of phosphate buffer. Into one side arm was placed a 0.4-ml amount containing $4\text{ }\mu\text{C}$ of $\text{NaH}^{14}\text{CO}_3$ (5.3 mc/mmmole); the other sidearm contained 0.3 ml of 20% trichloroacetic acid. The flasks were incubated with shaking in a Warburg apparatus at 30 C. One series of flasks was incubated under an atmosphere of 80% H_2 and 20% O_2 , with a duplicate series being incubated under 80% N_2 and 20% O_2 . After equilibrating for 15 min, the $\text{NaH}^{14}\text{CO}_3$ was tipped into the main compartment, and the reaction was allowed to proceed for 1 hr. The flasks were then removed from the water bath, and filter paper saturated with 40% KOH was placed into each center well. The flasks were stoppered, and the trichloroacetic acid was tipped into the main compartment. After allowing the remaining dissolved CO_2 to evolve, 1.0-ml samples were removed from the main compartment and assayed for radioactivity by using a Packard Tri-Carb liquid scintillation spectrometer. The scintillation fluid in each vial consisted of 3.0 ml of Triton X-100 and 6.0 ml of a toluene solution containing 0.4% PPO and 0.01% POPOP. The results were corrected for controls incubated under N_2 and O_2 .

Nitrogen determination. Inorganic nitrogen uptake was followed using Nessler's reagent. Cells were harvested by centrifugation at 2 C, washed, and suspended to 2.0 OD in media containing 0.01% $\text{MgSO}_4 \cdot 7\text{ H}_2\text{O}$, 0.001% $\text{Fe}(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{ H}_2\text{O}$, and 106 μmoles $(\text{NH}_4)_2\text{SO}_4$ in 0.04 M potassium phosphate buffer (pH 6.0). The cell suspension (50 ml) was added to flasks and incubated under an atmosphere of 70% H_2 , 20% O_2 , 10% CO_2 . Samples were withdrawn at the indicated time intervals and chilled in an ice bath to stop nitrogen uptake. The suspensions were then centrifuged at 2 C to remove the cells, and the clear supernatant fluid was assayed for nitrogen. Treatment of the suspensions with trichloroacetic acid rather than chilling yielded no significant difference.

RESULTS

The growth curves obtained when *H. eutropha* was cultured heterotrophically and mixotrophically in a glutamate-salts medium are shown in Fig. 1. Similar curves were obtained when acetate (4) or pyruvate (Fig. 2) was substituted for glutamate. With these substrates, heterotrophic growth rates and cell yields are about equal to the growth rates and yields during the initial phase of mixotrophic incubation. The fact that glutamate was exhausted at the initiation of the plateau period seen in Fig. 1 was confirmed by bioassay. Thus these three substrates probably do not allow simultaneous growth to occur, as was observed with some other organic substrates plus hydrogen (4, 18). The plateaus observed in the dicyclic curves shown in Fig. 1 and 2 indicate that

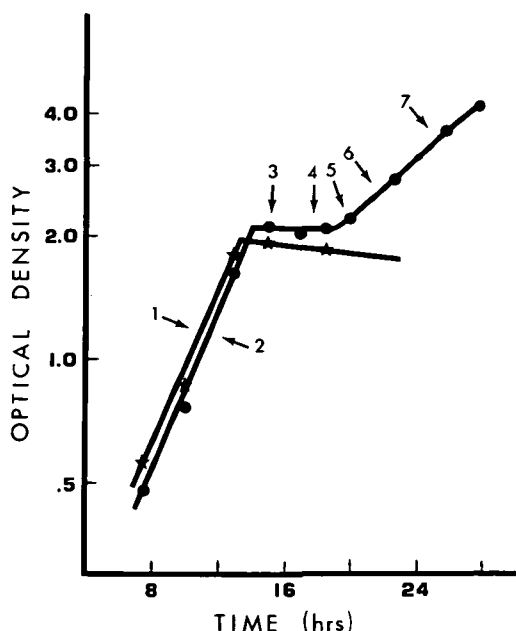


FIG. 1. Growth of *Hydrogenomonas eutropha* in mineral-salts media containing 0.2% *L*-glutamate. Incubation was under 70% H_2 , 20% O_2 , 10% CO_2 (●) or under air (★). The arrows indicate the points at which culture samples were withdrawn and assayed for their ability to incorporate ammonia (see Fig. 3). Ordinate is a logarithmic scale.

some enzyme(s) required for autotrophic growth is repressed by these substrates.

Cells grown heterotrophically in the presence of acetate or pyruvate did not possess significant RDPC activity (Table 1). Under dual substrate conditions, there also was no significant activity of RDPC until the acetate or pyruvate was consumed; however, as expected, enzyme activity was high during the second (autotrophic) phase of growth. During mixotrophic cultivation with pyruvate, the derepression of RDPC during the plateau period can be observed by comparing the points at which culture samples were withdrawn (Fig. 2) with their corresponding enzyme levels (Table 1).

The addition of acetate or pyruvate to an autotrophic extract had little effect on the RDPC activity (Table 2), providing evidence that acetate and pyruvate do not directly inhibit the enzyme.

Of the organic substrates tested, only acetate and pyruvate repressed RDPC during dual substrate incubation. Thus, although RDPC levels were low during heterotrophic growth on glutamate, the high enzyme levels present during the first phase of dual substrate incubation precluded the repression of this enzyme being responsible for the plateau observed with glutamate.

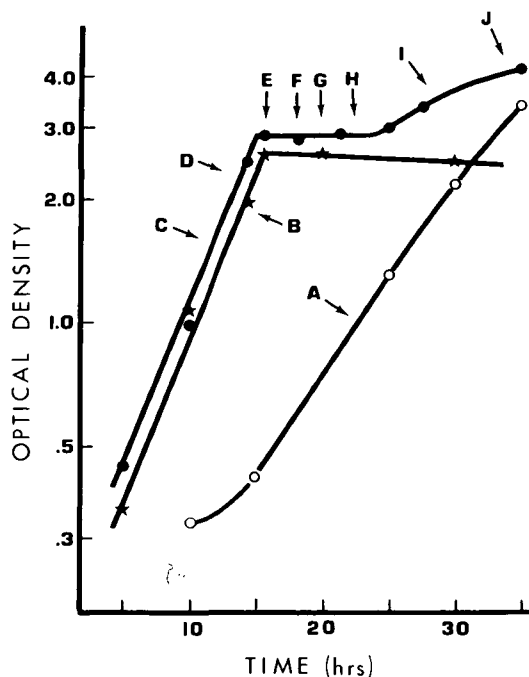


FIG. 2. Growth of *Hydrogenomonas eutropha* under autotrophic (○), heterotrophic (★), and mixotrophic (●) conditions. For heterotrophic and mixotrophic growth 0.3% pyruvate was added to the mineral-salts medium. Incubation was under air for heterotrophic growth and under 70% H_2 , 20% O_2 , 10% CO_2 for autotrophic and mixotrophic growth. The arrows indicate the points at which culture samples were withdrawn and assayed for ribulose diphosphate carboxylase (see Table 1).

The shaker speed at which cultures were incubated had no noticeable effect on RDPC levels when acetate or pyruvate was present. With cells grown either heterotrophically or mixotrophically on fructose, glutamate or alanine levels of RDPC were generally somewhat lower when rapid shaking was used. The activities in Table 1 were therefore determined with cultures incubated with slow shaking. The specific activity of RDPC from autotrophic cultures grown with slow shaking was 48.3 ± 5.1 units per mg protein. Autotrophic cultures grown with rapid shaking showed slightly lower specific activities (43.4 ± 5.6).

Dual substrate cultivation with fructose as the organic substrate gave a dicyclic growth curve lacking a plateau and similar to that seen with alanine (4). The high levels of RDPC present during mixotrophic cultivation with fructose or alanine are therefore in agreement with the growth patterns.

Cells cultured under conditions similar to those used for RDPC assay were also tested for their ability to assimilate labeled bicarbonate (Table

1). The results confirmed that heterotrophic and mixotrophic growth with pyruvate and acetate prevented autotrophic CO_2 fixation, whereas cells cultured mixotrophically in the presence of the other organic substrates allowed fixation to occur at high levels.

Since cells cultivated mixotrophically with glutamate contained a high level of RDPC, the plateau period after glutamate exhaustion must be explained in terms of some other function. One major difference between heterotrophic growth with glutamate and autotrophic growth is the assimilation of nitrogen. During autotrophic growth, *H. eutropha* must produce its organic nitrogen by the assimilation of ammonia, apparently to form glutamate. When grown on glutamate, inorganic nitrogen is not required and, in fact, may be somewhat inhibitory to growth (S:renkoski, unpublished data). It was reasoned, therefore, that the presence of glutamate may inhibit the assimilation of inorganic nitrogen during both heterotrophic and dual substrate incubation, and that the plateau after glutamate exhaustion may be the time required for the formation of a nitrogen-assimilating system. To investigate this possibility, cells harvested from various stages of the (glutamate) mixotrophic and heterotrophic growth curves (Fig. 1) were tested for their ability to take up nitrogen (Fig. 3). Cells harvested during heterotrophic growth or the first phase of mixotrophic culture showed little or no nitrogen uptake during the 80-min test period. Cells harvested from sequential points along the plateau showed increasing abilities to incorporate nitrogen, and a rate approaching the autotrophic level was reached during the second phase of the dicyclic growth curve. Cells grown heterotrophically or mixotrophically with pyruvate yielded nitrogen uptake rates similar to those of autotrophic cultures.

To demonstrate that the suppression of nitrogen uptake in cells grown on glutamate was not due to some accumulating inhibitor or other function of the culture medium, cells were harvested from various points along the dicyclic growth curve, washed, and inoculated into fresh autotrophic media. This procedure had no effect on the duration of the total lag before autotrophic growth commenced. Cells harvested previous to or at the beginning of the plateau required about 7 hr before growth began. Cells harvested at the end of the plateau showed little or no additional lag, whereas cultures from sequential points along the plateau showed decreasing lag times in fresh media. The total lag between the exhaustion or removal of glutamate, and the initiation of autotrophic growth was about 7 hr in all cases.

TABLE 1. *Relative levels of ribulose diphosphate carboxylase (RDPC) and CO₂ fixation during heterotrophic and mixotrophic growth of Hydrogenomonas eutropha*

Organic substrate	Atmosphere	Growth phase	H ¹⁴ CO ₂ Incorporation	RDPC activity
	Autotrophic ^a	Exponential ^b (A) ^c	1.00	1.00
Acetate	Air	Exponential	0.08	0
Acetate	Autotrophic	I ^d	0	0
Acetate	Autotrophic	II ^e	0.89	0.64
Pyruvate	Air	Exponential (B)	0	0.01
Pyruvate	Autotrophic	I (C)	0	0.02
Pyruvate	Autotrophic	I (D)	NA ^f	0.04
Pyruvate	Autotrophic	Plateau (E)	0	0.02
Pyruvate	Autotrophic	Plateau (F)	0.14	NA
Pyruvate	Autotrophic	Plateau (G)	NA	0.43
Pyruvate	Autotrophic	Plateau (H)	NA	0.64
Pyruvate	Autotrophic	II (I)	NA	0.96
Pyruvate	Autotrophic	II (J)	0.72	0.88
Fructose	Air	Exponential	0.55	0.64
Fructose	Autotrophic	I	0.89	0.77
Fructose	Autotrophic	II	0.85	1.00
Glutamate	Air	Exponential	0.29	0.36
Glutamate	Autotrophic	I	NA	0.74
Alanine	Air	Exponential	0.61	0.27
Alanine	Autotrophic	I	0.92	0.84

^a 70% H₂, 20% O₂, 10% CO₂.^b Since only one substrate was present, activities were determined with samples from the exponential portions of the standard growth curves.^c Letters A through J correspond to the points in Fig. 2 at which the samples were harvested.^d Harvested during the initial (mixotrophic) phase of dicyclic growth.^e Harvested during the second (autotrophic) phase of dicyclic growth.^f Not assayed.

DISCUSSION

A number of previous studies showed that autotrophic bacteria grown in the presence of organic compounds often contain decreased levels of autotrophic enzymes. In most cases these decreased enzyme levels were observed in the absence of the inorganic substrate; it was usually not determined if the enzymes would be similarly repressed in the presence of both organic and inorganic substrates. In one study, however, the facultative autotroph *Chromatium* contained low levels of RDPC during growth on pyruvate, but maintained high enzyme levels when both pyruvate and thiosulfate were present. The thiosulfate apparently prevented the repression of RDPC by pyruvate (8). Our results with *H. eutropha* demonstrate that the repression of RDPC by pyruvate or acetate occurs in the presence as well as the absence of the autotrophic atmosphere, and this repression is not due to a direct effect at the enzyme level.

During simultaneous cultivation with hydrogen and all tested organic substrates except glutamate, there was a correlation between the capacity of the cells to grow autotrophically and their RDPC activity. Substrates such as alanine

TABLE 2. *Effect of acetate and pyruvate on the activity of ribulose diphosphate carboxylase (RDPC) in an autotrophic extract of Hydrogenomonas eutropha*

Additions	RDPC (specific activity)
None	40.6
0.05% Pyruvate	33.0
0.1% Pyruvate	40.6
0.2% Pyruvate	42.4
0.1% Acetate	32.0
0.2% Acetate	30.2

and fructose which supported high levels of RDPC during the first phase of mixotrophic growth allowed autotrophic growth to commence rapidly when the organic substrate was consumed. With acetate and pyruvate the repression of RDPC during the first growth phase necessitated the synthesis of this enzyme before autotrophic growth could begin.

McFadden and Tu (15) reported that when *H. facilis* was grown heterotrophically on glutamate it contained approximately 3% of the autotrophic level of RDPC. We found, however, that *H. eutropha* grown on glutamate possessed

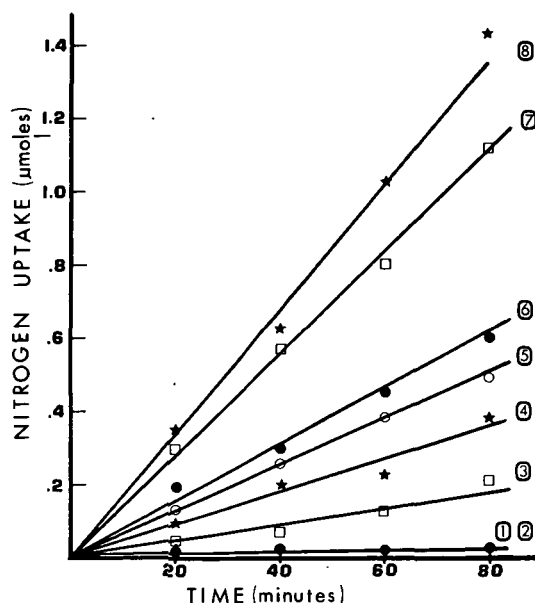


FIG. 3. Nitrogen uptake by *Hydrogenomonas eutropha* cells grown heterotrophically or mixotrophically on glutamate. The assays were performed under autotrophic conditions with cells which had been washed and resuspended in the mineral medium. Nitrogen uptake represents ammonia consumed per milliliter of OD 2.0 culture [0.64 mg (dry weight)/ml]. Curves 1 through 7 represent uptake by cells harvested at the corresponding points in the growth curves of Fig. 1. Curve 8 represents uptake by an autotrophically grown culture.

36% of the autotrophic level of RDPC, and, when grown mixotrophically on glutamate plus hydrogen, the RDPC level increased to 74%. Thus a repression of RDPC by glutamate could not account for the plateau observed in the growth curves during dual substrate incubation. However, heterotrophic and mixotrophic growth on glutamate does directly or indirectly interfere with nitrogen uptake or assimilation, and this deficiency may be responsible for the temporary inability to grow autotrophically.

Of the major nitrogen assimilating catalysts, only glutamate dehydrogenase has been found in *H. eutropha* (Strenkoski, unpublished data). Its properties are similar to those reported for *Thiobacillus novellus* (12), and it catalyzes both the synthesis and degradation of glutamate in vitro. Since cells grown on glutamate possess high levels of glutamate dehydrogenase which could presumably function biosynthetically after glutamate exhaustion, the control point for nitrogen assimilation in *H. eutropha* is probably not the assimilating enzyme itself but may be either the nitrogen uptake mechanism or the synthesis of 2-oxoglutarate. Heterotrophic or mixotrophic

growth on glutamate might therefore suppress either of these processes.

The correlation between the relative activities of RDPC and resting cell CO_2 fixation, using cells grown both heterotrophically and mixotrophically, strongly implicates RDPC as the major catalyst for knallgas-supported CO_2 fixation in *H. eutropha*. This correlation can be compared with the parallel levels of RDPC and cell-free reductive CO_2 fixation observed with *H. facilis* (15) and suggests a similarity between the pathways of CO_2 assimilation and their regulation in these two species.

ACKNOWLEDGMENT

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Autotrophic and Heterotrophic Metabolism of *Hydrogenomonas*

I. Growth Yields and Patterns Under Dual Substrate Conditions

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Auxotrophic mutants of *Hydrogenomonas eutropha* and *H. facilis* requiring utilizable amino acids were employed to demonstrate the simultaneous utilization of H_2 and an organic substrate for growth. The ratio of the cell yields under dual substrate conditions compared to heterotrophic conditions indicated the relative contributions of the autotrophic and heterotrophic systems to the growth of the organism. Wild-type *H. eutropha* grown under simultaneous conditions exhibited a dicyclic growth pattern, the first cycle representing either heterotrophic or simultaneous growth and the second cycle representing autotrophic growth. The duration of the changeover period was either very short with no plateau or long with a plateau up to 8 hr, depending upon the organic substrate. The growth rate under simultaneous conditions with some organic substrates was faster than either the autotrophic or heterotrophic rate, but was not the sum of the two rates. The data suggest that, in the presence of both organic and inorganic substrates, heterotrophic metabolism functions normally but autotrophic metabolism is partially repressed.

All of the known hydrogen-oxidizing autotrophs are capable of heterotrophic growth on a wide variety of organic substrates, including organic acids, sugars, and amino acids. The capacity to utilize these organic substrates individually as sole carbon and energy sources can be employed to distinguish the various strains and species within this group. Two of the most extensively studied species, *Hydrogenomonas facilis* and *H. eutropha*, can be readily distinguished in this manner (Hogan, M.S. Thesis, The Catholic University of America, Washington, D.C., 1965), as well as by the difference in their growth rates, *H. facilis* being conspicuously slower under both autotrophic and heterotrophic conditions. Three other strains which have received a great deal of attention from Schlegel and his co-workers are *Hydrogenomonas* H1, H16, and H20 (14); according to substrate utilization and phage sensitivity, these strains are closely related to *H. eutropha* (Edmister, unpublished data).

Besides the taxonomic significance, much of the research on organic substrates has been concerned with possible relationships to autotrophic physiology. Thus, it has been shown that *H. facilis* (10), *H. eutropha* (Bovell, Ph.D. Thesis, University of California, Davis, 1957), and strain H16 (7) can assimilate carbon dioxide during growth on a number of organic substrates. On the other hand,

it has been amply demonstrated with a number of hydrogenomonads that growth on organic substrates can reduce or eliminate the hydrogen-oxidizing and CO_2 -fixing systems which are vital to autotrophic metabolism (1, 6, 8, 12, 13, 15). The response apparently depends upon the specific organic substrate, bacterial strain, and gas atmosphere employed.

The simultaneous oxidation of H_2 and an organic substrate was demonstrated by Kluyver and Manten (8) and Wilson et al. (15) with *H. ruhlandii* and *H. facilis*, respectively; however, the derivation of useful energy from both oxidations does not necessarily follow since, with some hydrogenomonads, the activity of the hydrogen-oxidizing system is not dependent upon the presence of CO_2 . With *Hydrogenomonas* H1, however, a drastic increase in hydrogen oxidation during CO_2 assimilation has been reported (2), suggesting that phosphorylation is strongly coupled to hydrogen oxidation in this organism. In agreement with this, the closely related *H. eutropha* has exhibited increased cell densities in the presence of H_2 and lactate as compared to growth in lactate alone (Goodman and Riittenberg, Bacteriol. Proc., p. 112, 1964).

In this paper, we provide evidence for the simultaneous utilization of H_2 and organic substrates for the growth of *H. facilis* and *H. eutropha*.

Auxotrophic mutants requiring utilizable amino acids were employed to enable precise determination of the cell yield at the point of organic substrate exhaustion. This method also eliminates the possibility of growth by means of the sequential utilization of the inorganic and organic substrates. The results with the mutants were correlated with organic substrate consumption and growth patterns of wild-type *H. eutropha* and enabled us to conduct a comparative and quantitative study of substrate utilization under simultaneous conditions.

MATERIALS AND METHODS

Organisms. Wild-type *H. eutropha* was kindly supplied by Roy Repaske, National Institutes of Health, Bethesda, Md. Two mutant strains of *H. eutropha*, designated A12 and A14, which require histidine and tryptophan, respectively, were derived from the wild type. A mutant of *H. facilis*, designated as strain 4A2, which requires phenylalanine, tryptophan, and *p*-aminobenzoate (PABA) was described previously (5).

Selection of mutants. Mutant strains A12 and A14 were obtained by use of the following modification of the penicillin technique (4, 9). Cultures of wild-type *H. eutropha* in the exponential growth phase were irradiated with ultraviolet light to a survival of 2×10^{-3} . Samples from the irradiated cultures were inoculated into Tryptic Soy Broth (Difco) and incubated overnight at 30 C. The cultures were washed twice with 0.85% saline and were suspended in saline. Flasks containing autotrophic medium were inoculated with the washed cells to just visible turbidity (about 10^7 cells/ml), and penicillin G was added to a final concentration of 20,000 units/ml. The cultures were incubated overnight at 30 C under autotrophic conditions, then penicillinase (Difco) was added to a concentration of 1,000 units/ml, and the cultures were reincubated for 30 min to inactivate the penicillin. Dilutions of the cultures were spread onto plates containing Tryptic Soy Agar (Difco), and auxotrophic clones were identified by replica-plating and auxanography.

Media and cultural conditions. The mineral-salts solution used for the growth studies was a modification of that described by Bongers (3); it contained 0.1% $(\text{NH}_4)_2\text{SO}_4$ and 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 0.04 M potassium phosphate buffer, pH 6.65. Filter-sterilized $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ was added to a final concentration of 0.001%. Organic substrates were added to the specified concentrations. Autotrophic and simultaneous cultures were incubated under a gas atmosphere of 70% H_2 , 20% O_2 , and 10% CO_2 . The gas mixture was continuously supplied through a manifold from a gas reservoir and was maintained at slightly greater than atmospheric pressure. Heterotrophic controls contained N_2 in place of H_2 and yielded results very similar to cultures incubated in air. Growth was monitored by following the increase in cultural density either by use of (i) a Bonet-Maury and Jouan biophotometer (Jouan, Paris) which automatically records changes in the transmission of

light through six bacterial cultures at 2-min intervals or (ii) periodically removing samples from 100-ml cultures in baffled 250-ml Erlenmeyer flasks and reading the optical density (OD) at 540 m μ in a Spectronic-20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.). Percentage transmittance readings obtained with the biophotometer were converted to their corresponding OD values and were multiplied by 0.49 to conform to OD values obtained with 1-cm cuvettes in the Spectronic-20 colorimeter (an OD of 1.0 corresponds to a cellular dry weight of 0.32 mg/ml). Virtually identical growth curves were obtained with both methods under similar conditions, except that, as described below, the recording biophotometer enabled us to discern slight fluctuations in the growth pattern. Flask cultures were incubated at 30 C with shaking on a model G25 incubator shaker (New Brunswick Scientific Co., New Brunswick, N.J.). Incubation in the biophotometer was conducted at 28 to 32 C with agitation by magnetic oscillation.

Tryptophan measurements. Residual tryptophan remaining during heterotrophic and simultaneous growth in a tryptophan-salts medium was determined by bioassay. *Lactobacillus plantarum* ATCC 8814 and Tryptophane Assay Medium (Difco) were employed for the assay. Samples taken from flask cultures at the stated cell densities were immediately placed in an ice bath and were then centrifuged at 4 C to sediment the cells. The supernatant fluids were filter-sterilized by use of Swinnex 13 filter units (Millipore Corp., Bedford, Mass.) with a pore size of 0.45 μ and were then assayed for tryptophan.

RESULTS

Growth yields with amino acid auxotrophs. Mutant strains of *H. eutropha* requiring histidine, tryptophan, methionine, cysteine, or phenylalanine plus tryptophan were isolated by use of the methods described. Of these, strains A12 and A14 were employed for the growth yield studies because of their ability to utilize histidine and tryptophan, respectively, as sole carbon and energy sources (Hogan, M.S. Thesis, The Catholic University of America, Washington, D.C., 1965). The maximal cell densities attained with increasing concentrations of each amino acid under either heterotrophic or simultaneous conditions are shown in Fig. 1. With both strains, greater cell yields were obtained under simultaneous conditions as compared to heterotrophic conditions, demonstrating the sparing effect of the autotrophic metabolism on the utilization of either amino acid. The slope of the line drawn through the growth yields under simultaneous conditions can be compared to the slope of the heterotrophic yields for each amino acid. The ratio of the simultaneous to heterotrophic slopes indicates the extent to which autotrophic metabolism is contributing to the growth of the organism under simultaneous conditions.

Increased growth yields were also noted when

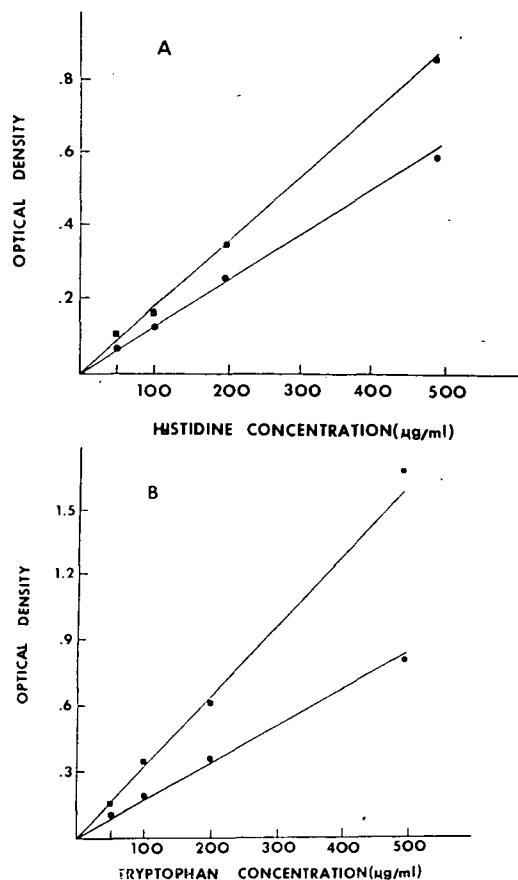


FIG. 1. Maximal cell yields of *Hydrogenomonas eutropha* auxotrophs grown in increasing concentrations of the required L-amino acid. The gas mixtures present were 70% H₂, 20% O₂, and 10% CO₂, ■; 70% N₂, 20% O₂, and 10% CO₂, ●.

H. facilis 4A2 was grown in the presence of a utilizable organic substrate plus hydrogen (Fig. 2). When both phenylalanine and hydrogen were available, the ratio of the simultaneous to heterotrophic yields was 1.35:1, indicating that autotrophic metabolism accounts for about 25% of the culture's growth during dual substrate incubation.

Growth patterns with wild-type *H. eutropha*. With the system described above, growth yield comparisons can only be performed with auxotrophic mutants requiring growth factors which are utilizable as carbon and energy sources. To expand these studies, it was necessary to determine the cell densities at which other substrates are exhausted during both simultaneous and heterotrophic cultivation. When wild-type *H. eutropha* was grown under simultaneous conditions, several growth patterns were possible,

depending upon the organic substrate and the environmental conditions. When the organic substrate was tryptophan and conventional methods were employed for measuring OD, a typical growth curve was observed. Thus, if the wild-type organism was functioning in a manner similar to strain A14, a rapid unobservable changeover from simultaneous to autotrophic growth would have occurred at a point corresponding to the simultaneous cell yield of the mutant culture at the same tryptophan concentration. However, when the cell density of a simultaneous culture containing 100 μg of tryptophan per ml was closely monitored with the Jouan biophotometer, a deviation from the standard growth pattern was observed at an OD of approximately 0.33 (Fig. 3). This agrees with the maximal OD of 0.33 observed in mutant strain A14 (Fig. 1B) with the same tryptophan concentration and suggests that this irregularity in the growth curve corresponds to the point of tryptophan exhaustion. The maximal cell densities of both wild-type and tryptophan-requiring strains also agree well under heterotrophic conditions.

If the irregular point in the growth curve of wild-type *H. eutropha* actually corresponds to the

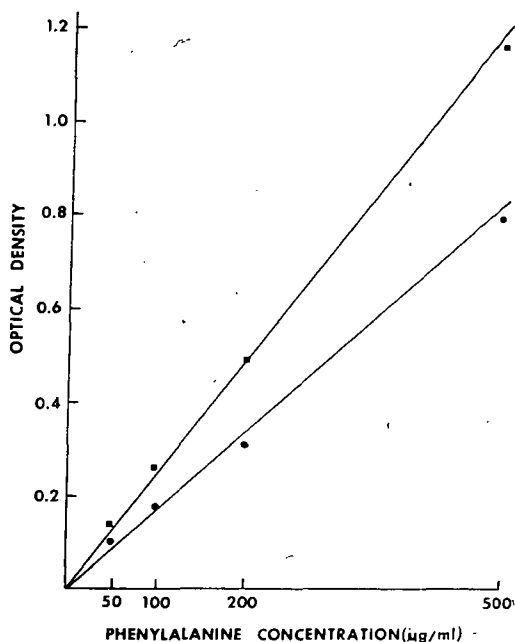


FIG. 2. Maximal cell yields of *Hydrogenomonas facilis* 4A2 grown in increasing concentrations of a required and utilizable amino acid, L-phenylalanine. L-Tryptophan and PABA, which are also required but are not utilizable for carbon and energy, were present at concentrations (per ml) of 5 μg and 1 mμg, respectively. For explanation of symbols, see Fig. 1.

point of tryptophan exhaustion and if this irregularity is observable with other utilizable organic substrates under simultaneous conditions, then a simple method would be available for determining and comparing the ratios of simultaneous to heterotrophic growth yields with a number of organic substrates. The extent of autotrophic metabolism with each substrate could be calculated from these values.

To confirm that the irregularity in the growth curve was due to the exhaustion of tryptophan, the amount of tryptophan remaining in the extracellular medium at different cell densities was determined by bioassay. Under heterotrophic conditions (see Fig. 3, curve B), a direct correlation was noted between the percentage of tryptophan utilized and the percentage of the maximal culture density. At an OD of 0.13 (which is 72% of the maximal heterotrophic cell density of 0.18), 71% of the tryptophan had been utilized. A similar correlation between the percentage of tryptophan utilized and percentage of culture density at the presumed changeover point (OD, 0.33) was also seen under simultaneous conditions (Table 1), but the corresponding OD values were pro-

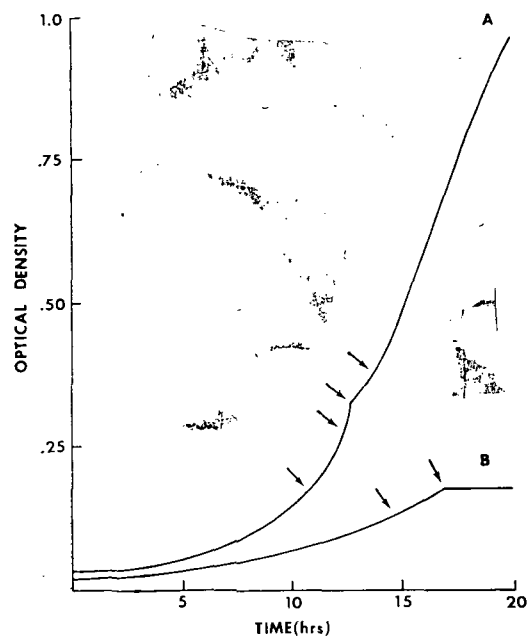


FIG. 3. Growth of *Hydrogenomonas eutropha* in mineral-salts medium containing 100 µg/ml of tryptophan. Growth was monitored by use of a Jouan biophotometer. The culture producing curve A was incubated under a gas mixture of 70% H_2 , 20% O_2 , and 10% CO_2 . For curve B, the gas mixture was 70% N_2 , 20% O_2 , and 10% CO_2 . The arrows indicate the culture densities at which samples were withdrawn from flasks and assayed for tryptophan. (See Tables 1 and 2.)

TABLE 1. Tryptophan consumption during simultaneous growth of *Hydrogenomonas eutropha* in mineral-salts medium containing 100 µg/ml tryptophan

OD	Percentage of tryptophan utilized ^a	Percentage of OD 0.33 ^b
0.18	53	55
0.28	85	85
0.33	95	100
0.38	100	115

^a Calculated by subtracting the percentage of tryptophan remaining from 100%.

^b This is the suspected changeover point (see curve A in Fig. 3).

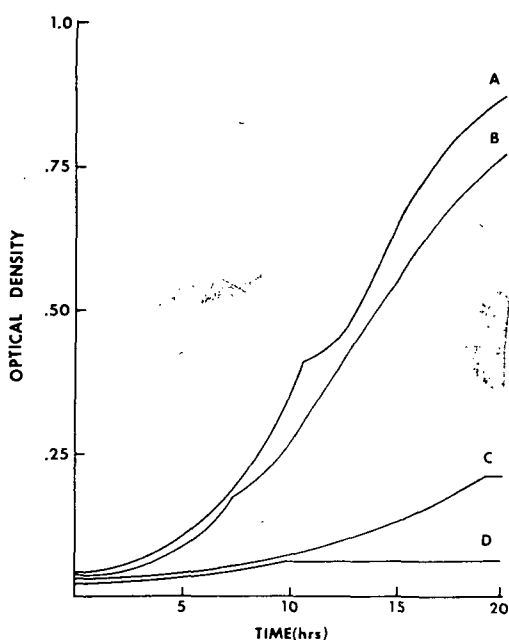


FIG. 4. Growth of *Hydrogenomonas eutropha* in mineral-salts media containing either 50 µg/ml (curves B and D) or 200 µg/ml (curves A and C) of L-alanine. Growth was monitored by use of a Jouan biophotometer. The cultures producing curves A and B were incubated under 70% H_2 , 20% O_2 , and 10% CO_2 . Curves C and D were obtained with cultures incubated under 70% N_2 , 20% O_2 , and 10% CO_2 .

portionately higher. Thus, at an OD of 0.18, when all the tryptophan had been consumed and growth had ceased under heterotrophic conditions, almost 50% of the tryptophan remained when the autotrophic atmosphere was also present. This finding predicts that, under simultaneous conditions, the cell density at the point of tryptophan exhaustion should be almost double the value under heterotrophic conditions (the data in Fig. 1B and Table 1 agree).

Simultaneous growth patterns obtained with a number of organic substrates exhibit dicyclic curves to a greater or lesser extent. Figure 4 shows the simultaneous growth patterns obtained with two concentrations of alanine. Dicyclic curves are seen at both concentrations and the break point in each curve can be compared to the heterotrophic yield with an equal amount of alanine.

In contrast to the extremely brief changeover periods in the simultaneous growth patterns with either tryptophan or alanine, other substrates yielded growth curves with longer changeover times (or plateaus) which varied in length up to approximately 8 hr. When the organic substrate was acetate, the growth curve was of this type (Fig. 5); the cell yield under simultaneous conditions was very similar to the heterotrophic yield, indicating that the heterotrophic system alone is responsible for growth. Similar results were observed with pyruvate and glutamate; long plateaus and little or no increase in yields were noted under simultaneous conditions.

Table 2 lists the growth rates for cultures of *H. eutropha* growing either simultaneously or heterotrophically with a number of organic substrates. Since the autotrophic rate under the same condi-

TABLE 2. Relationship between growth rate and cell yield of *Hydrogenomonas eutropha* during simultaneous and heterotrophic incubation

Organic substrate	Growth rate (doublings/hr)			Cell yield ^a
	Simultaneous	Heterotrophic	Simultaneous/Heterotrophic	Simultaneous/Heterotrophic
Histidine	0.34	0.22	1.5	1.4
Tryptophan	0.37	0.21	1.8	1.9
Alanine	0.36	0.17	2.1	2.0
Acetate	0.50	0.50	1.0	1.0

^a Calculated from data shown in Fig. 1A, 1B, 3, 4, and 5. Figures 1B and 3 give a value of 1.9 with tryptophan as the organic substrate. Yields with alanine were calculated from the 200 μ g/ml of alanine curves in Fig. 4.

tions is 0.30, it is evident that the simultaneous growth rates with histidine, alanine, or tryptophan are faster than either the autotrophic or heterotrophic rates; however, the simultaneous rates are not equal to the sum of the autotrophic and heterotrophic values. A relationship between the growth rates during simultaneous and heterotrophic growth and the cell yields under the same conditions can be observed from the data in Table 2. When the simultaneous growth rate is faster than the heterotrophic rate, a proportionate increase in cell yield is seen.

DISCUSSION

The use of amino acid auxotrophs to demonstrate the simultaneous utilization of an organic and an inorganic substrate for growth offers several advantages. The requirements are such that growth immediately ceases upon substrate exhaustion, making quantitation quite precise. The requirements also preclude the possibility that the increased yields under simultaneous conditions result from sequential rather than simultaneous utilization of the inorganic and organic substrates. The correlation seen in Table 1 between tryptophan consumption and culture density under dual substrate conditions further demonstrates a continuous and simultaneous utilization of both substrates. These results confirm and extend the observations of Goodman and Rittenberg (Bacteriol. Proc., p. 112, 1964) concerning the growth of wild-type *H. eutropha* under dual substrate conditions. These investigators noted equivalent growth rates under simultaneous and heterotrophic conditions which were more rapid than the autotrophic rate. Although we observed similar results with certain organic substrates, e.g., pyruvate or acetate, other sub-

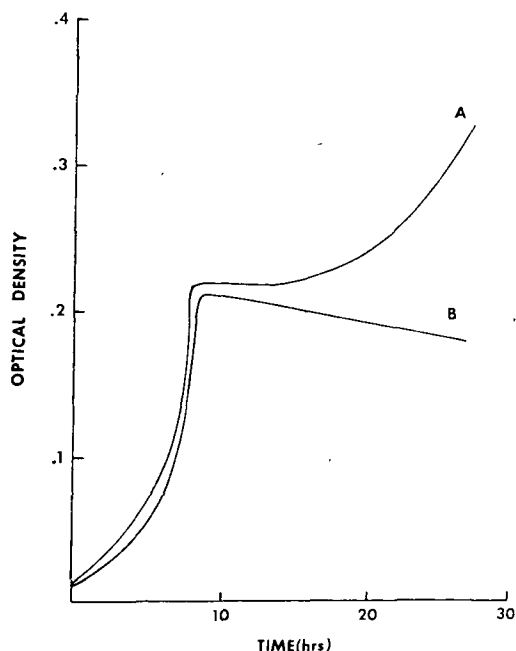


FIG. 5. Growth of *Hydrogenomonas eutropha* in mineral-salts medium containing 200 μ g/ml of acetate. Growth was monitored by use of a Jouan biophotometer. Culture A was incubated under 70% H_2 , 20% O_2 , and 10% CO_2 . Culture B was incubated under 70% N_2 , 20% O_2 , and 10% CO_2 .

strates allowed more rapid growth under dual substrate conditions than with either substrate alone. This was true of all tested organic substrates which provide heterotrophic growth rates that are slower than the autotrophic rate. Since those organic substrates permitting increased growth rates also produced greater cell yields in the presence of hydrogen, some aspect of autotrophic metabolism must be contributing to the growth of the organism under simultaneous conditions.

The observed relationship between simultaneous/heterotrophic growth rates and simultaneous/heterotrophic yields suggests that the heterotrophic system functions at a similar level during both simultaneous and heterotrophic growth. Thus, with tryptophan, the ratio of the growth rates is 1.8:1. If, under simultaneous conditions, tryptophan is being utilized at the heterotrophic rate, then the cell yield at the point of tryptophan exhaustion under simultaneous conditions should be about 1.8 times that observed under heterotrophic conditions. The results in Table 2 support this idea. Since the growth rates in the presence of both organic and inorganic substrates are not the sum of the individual rates, it appears that autotrophic metabolism is not fully functional under simultaneous conditions. If the contribution to simultaneous growth was proportional to the individual autotrophic and heterotrophic growth rates, then the growth yields with organic substrates such as tryptophan, alanine, or histidine would be much greater than those observed.

The dicyclic growth curves seen in Fig. 3, 4, and 5 resemble the diauxic pattern noted by Monod (11); however, the cause may be quite different. Rather than the two phases representing the sequential utilization of two substrates, the first growth cycle in Fig. 3 and 4 represents the simultaneous utilization of two substrates until one is exhausted, followed by a second period of growth on the remaining substrate (hydrogen). When tryptophan, alanine, or histidine is present, no plateau is observed; the second growth phase begins immediately after exhaustion of the organic substrate without the induction period characteristic of the diauxic phenomenon. This changeover point is usually missed with conventional methods for monitoring growth. Even with the biophotometer, the point of changeover is occasionally unobservable; this means that the changeover period must be no longer than about 10 min in duration.

When the organic substrate is glutamate, pyruvate, or acetate, a plateau of 5 to 8 hr duration is observed after dual substrate incubation. This plateau presumably represents the time required

for the induction of some aspect of the autotrophic system. In contrast to most other hydrogenomonads, the hydrogen-oxidizing system in *H. eutropha* is constitutive. Even after growth on substrates, which produce a long plateau (e.g., glutamate), the hydrogenase activity of this species is similar to autotrophic levels (Bovell, Ph.D. Thesis, University of California, Davis, 1957; Stukus, M.S. Thesis, The Catholic University of America, Washington, D.C., 1966); thus, the plateau must be the induction period for some other autotrophic function. In the case of pyruvate and acetate, we believe it represents the synthesis of ribulose diphosphate carboxylase; whereas with glutamate, since the ribulose diphosphate carboxylase activity during *simultaneous incubation* is at the autotrophic level, the plateau probably represents the synthesis of an aminating enzyme (Stukus, *unpublished data*). Whenever an organic substrate producing a plateau is present little or no increased cell yield is observed, suggesting that the complete autotrophic system is necessary for hydrogen oxidation to yield useful energy.

The presence or absence of a plateau during dual substrate incubation may be a general indication of whether a particular organic substrate represses some aspect of the autotrophic physiology of a hydrogenomonad. Those substrates yielding no plateaus apparently allow a functional autotrophic system during simultaneous incubation and might be expected to show increased growth yields. Similar effects are noted when cultures grown heterotrophically are used as inocula for autotrophic cultivation. Cultures grown on substrates showing plateaus undergo long lags before autotrophic growth commences, whereas cultures from alanine, histidine, or tryptophan media are capable of immediate autotrophic growth (*unpublished data*). Therefore, the method of inoculum growth has a predictable effect on simultaneous growth yields; cultures grown on substrates which repress the autotrophic system will show decreased yields under dual substrate conditions, whereas an autotrophically grown inoculum will allow greater simultaneous yields.

There is still little evidence as to what simultaneous growth actually involves. Does the oxidation of hydrogen simply spare the utilization of the organic substrate for energy, thereby making it relatively more important as a carbon source, or is hydrogen oxidation strongly coupled to CO₂ assimilation during simultaneous growth, so that the complete autotrophic system lessens the need for the organic compound for both energy and carbon? The rapid changeover from simultaneous to autotrophic growth noted with those

organic substrates showing increased growth yields suggests that, under these conditions, the complete autotrophic system is functioning during simultaneous growth. Since those substrates which yield plateaus during simultaneous incubation show no increased growth yields under the same conditions, an obligatory link between hydrogen oxidation and the repressed autotrophic system seems likely with *H. eutropha*. This would agree with the strong coupling of hydrogen oxidation and CO₂ assimilation noted with strain H1 (2). The utilization pattern of isotopically labeled organic substrates and CO₂ supplied during dual substrate incubation should answer these questions.

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pH-Conditional, Ammonia Assimilation-Deficient Mutants of *Hydrogenomonas eutropha*: Isolation and Growth Characteristics¹

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Two mutants of the facultative autotroph *Hydrogenomonas eutropha* were isolated by using a modified penicillin selection method. The mutation involved was unusual in that its effect on cellular growth was conditional with regard to extracellular *pH* and the type of substrate employed. Growth of both mutants was abnormal under autotrophic conditions and during heterotrophic cultivation in the presence of organic substrates which lacked an amino group. Abnormal growth was characterized by linear growth rates which were low at *pH* 6.0 and moderate at *pH* 7.2. In contrast, growth of the mutants was normal on most amino acids. Those substrates yielding abnormal growth were oxidized at normal rates by the mutants, indicating the mutation did not impair their uptake or metabolism. The data suggest that the mutants are defective in their ability to assimilate inorganic nitrogen into organic forms, and this defect is strongly influenced by *pH*.

Because of its ability to grow rapidly under autotrophic, heterotrophic, or mixotrophic conditions (2, 5) *Hydrogenomonas eutropha* affords an excellent means of comparing autotrophic and heterotrophic metabolism. One method which could be employed for such a comparative study would involve the use of obligate heterotrophic mutants of such an organism. However, while employing a technique to isolate such mutants two isolates were obtained which seemed to be "*pH*-conditional" with respect to autotrophy, i.e., they grew autotrophically at neutral to alkaline *pH* but not at acid *pH*. The affected system was eventually shown to involve nitrogen metabolism rather than a deficiency in some aspect of autotrophic metabolism.

The purpose of this communication and the one which follows (6) is to describe the isolation and properties of these mutants and the implications of these studies for nitrogen transport and metabolism in *H. eutropha* and possibly other bacteria.

MATERIALS AND METHODS

Media and cultural conditions. The mineral-salts solution used for the growth studies contained 0.1%

(NH_4)₂SO₄, 0.01% MgSO₄·7H₂O, and 0.001% Fe·(NH₄)₂(SO₄)₂·6H₂O (filter-sterilized) in 0.04 M potassium phosphate buffer at the desired *pH*. For autotrophic incubation, cultures were placed under an "autotrophic atmosphere" consisting of 70% H₂, 20% O₂, and 10% CO₂. For heterotrophic growth, individual organic substrates were added to a final concentration of 0.2% and incubation was under air. For solid media agar (Difco) was added to a final concentration of 1.5%. Growth was monitored as previously described (2) either by use of a Bonet-Maury and Jouan biophotometer (Jouan, Paris) or by reading the optical density (OD) of samples at 540 mμ in a Spectronic-20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.). An OD of 1.0 corresponds to a cellular dry weight of 0.65 mg/ml. All cultures were incubated at 30°C.

Selection of mutants. The two mutant strains, He4 and He8, were two isolates derived from wild-type *H. eutropha* (HeW) by use of the following modification of the penicillin technique (1, 3). Cultures of HeW were grown in 1.5% Tryptic Soy Broth (TSB, Difco) to the late exponential growth phase, harvested by centrifugation, washed twice with potassium phosphate buffer (0.04 M, *pH* 7.2), and resuspended in the same buffer. The cell suspensions were irradiated with ultraviolet light to a survival of 1.9×10^{-3} . Samples from the irradiated cell suspensions were inoculated into mineral-salts medium (*pH* 6.8) containing 0.2% L-monosodium glutamate and incubated until an increase in turbidity occurred (16 to 20 hr). The cultures were centrifuged, washed twice with buffer, resuspended in buffer, and starved for 1 hr with shaking. Cells were inoculated to just visible turbidity into flasks containing mineral-salts medium (*pH* 7.2) plus 40,000 units of penicillin G per

¹ This report was taken from a dissertation submitted by L. F. S. to Catholic University in partial fulfillment of the requirements for the Ph.D. degree. It was presented in part at the 69th Annual Meeting of the American Society for Microbiology, Miami Beach, Fla., 4-9 May 1969.

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ml and incubated overnight under an autotrophic atmosphere. Penicillinase (Difco) was then added to a concentration of 2,000 units/ml, the suspensions were incubated for 30 min to inactivate the penicillin, and dilutions of the suspensions were spread onto glutamate-agar plates. Clones not able to grow autotrophically were identified by successive replica plating onto autotrophic, phenylalanine-, and glutamate-agar at pH 6.0.

Determination of the rates of reversion of mutants to prototrophy. The method used was that of Luria and Delbruck (4). Mutant cells were grown in test tubes (18 by 150 mm) containing 2.0 ml of TSB. In this medium mutant and wild-type cells grow at the same rate. Total population counts were performed on Tryptic Soy Agar. Revertants were determined by using sodium pyruvate-agar at pH 6.0.

Manometric experiments. Conventional manometric techniques (7) were used to measure the rate of oxidation of various substrates. Oxidation rates were expressed in terms of microliters of gas consumed per hour per milligram (dry weight) of cells. The gas phase was air when organic substrates were supplied. Hydrogen oxidation was measured by omitting the organic substrate and providing a gas mixture containing 70% H₂, 20% O₂, and 10% N₂. The reactions were carried out at 30 C in shaking Warburg flasks with KOH in the center well.

RESULTS

The two mutant strains which were isolated, designated He4 and He8, were suspected of being strict heterotrophs because they grew well on phenylalanine- and glutamate-agar but did not grow on autotrophic agar. They were exposed to bacteriophage specific for *H. eutropha* (Edmister and DeCicco, unpublished data) to prove that the isolated clones were mutants of *H. eutropha* and not contaminants. Both He4 and He8 were lysed by the phage. Their relatedness to *H. eutropha* was also confirmed by their ability to revert to prototrophy. The revertant cultures from both strains were physiologically identical to *H. eutropha* including the ability to grow autotrophically by using hydrogen. Phenylalanine and glutamate were the organic substrates employed during the isolation and screening procedure because these amino acids support rapid growth of *H. eutropha*. The mutants were also tested for growth on fructose-agar to confirm that they were obligate heterotrophs, and when both strains failed to grow it was obvious that they were not lacking simply the capacity to grow autotrophically. While studying the nutritional characteristics of the mutants, the pH of the media was changed from 6.0 to 7.2, and it was observed that this change allowed He4 and He8 to grow on all four simple media: glutamate-, phenylalanine-, fructose-, and autotrophic-agar. Wild-type *H. eutropha* He4 and He8 were then tested for their ability to grow on a number of different

substrates at pH 6.0 and 7.2. All substrates which supported growth of HeW allowed the mutant strains to exhibit either of two growth patterns; on some substrates they grew at both pH values at rates similar to the wild type (normal growth). With other substrates they grew more slowly than the wild type at pH 7.2 and yielded little or no growth at pH 6.0 (abnormal growth). The results are given in Table 1. Growth of HeW, He4, and He8 at pH 6.0 and 7.2 on glutamate, where the deficiency of the mutants is not expressed, is seen in Fig. 1. Growth on pyruvate, which allows phenotypic expression of the mutation, is shown in Fig. 2. HeW exhibited identical logarithmic growth rates at both pH values on pyruvate. In contrast, the growth rates of both mutant strains were strongly influenced by the pH of the medium. After initial periods of logarithmic growth, growth rates of He4 and He8 became linear; the duration of logarithmic growth and the magnitude of linear growth were much greater at the higher pH. The poor growth of the mutants at pH 6.0 explains why growth appeared negative at this pH on fructose- and autotrophic-agar.

The physiological characteristics of mutant strains He4 and He8 were qualitatively identical, although some differences in growth rates were detectable (Fig. 1 and 2). During the growth and nutritional studies, reversions to the wild phenotype occurred with both strains; however, although revertants were observed frequently with strain He4, they were encountered only rarely with He8. These observations suggested that the two mutant strains are genetically nonidentical although the same functional locus is probably affected. To confirm this difference and to measure their stability, the reversion rates of strains He4 and He8 were determined by using the second method of Luria and Delbruck (4). He4 reverted to the wild type at a rate of 2.1×10^{-7} mutations per cell per generation, whereas He8 showed a reversion rate of 1.3×10^{-10} . The relatively high rate of reversion by strain He4 revealed why revertants were always present when He4 was grown to heavy densities and indicated that this strain might prove unsatisfactory for further physiological or biochemical studies. Most additional studies were therefore performed with strain He8 and, although cultures were routinely assayed for revertants, no problems were encountered with this strain.

The data in Table 1 demonstrate that the mutation produces abnormal growth on all tested substrates lacking an amino group. In contrast, the mutants grew normally on all amino acids tested except alanine, serine, threonine, and tryptophan. These results suggest that the mutation is affecting nitrogen metabolism, probably by inter-

TABLE 1. Growth patterns of mutant strains He4 and He8 using various energy sources^a

Normal growth ^b	Abnormal growth ^c
L-Glutamate	D-Fructose
L-Phenylalanine	D-Glucose
L-Aspartate	
L-Proline	Pyruvate
L-Valine	Fumarate
L-Histidine	Succinate
L-Leucine	Acetate
L-Isoleucine	Citrate
L-Lysine	2-Oxoglutarate
Tryptic Soy Broth	DL-Lactate
	Oxalacetate
	L-Malate
	L-Alanine
	L-Tryptophan
	L-Serine
	L-Threonine
	Autotrophic (H ₂ , O ₂ , CO ₂)
	Glycerol

^a Wild-type *H. eutropha* (HeW) can utilize all of the listed substrates as sole carbon and energy sources with the exception of glucose. For the glucose test, glucose-utilizing mutants were isolated and used.

^b Normal growth is defined as being similar to growth of HeW.

^c Abnormal growth refers to little or no growth at pH 6.0 and slower growth than HeW at pH 7.2.

fering with the assimilation of inorganic nitrogen into organic forms. The amino acids, with the four exceptions noted, can apparently satisfy the requirement for amino nitrogen and thereby allow normal growth.

Further evidence that a lack of amino nitrogen was responsible for the poor growth of the mutants at pH 6.0 is shown in Fig. 3. The presence of a low concentration of glutamate greatly enhanced the growth of He8 in a pyruvate medium containing ammonium sulfate. The increased growth rate was not due solely to the utilization of glutamate as a carbon and energy source, since this substrate was present in limiting concentration. The pyruvate present must have been supplying the bulk of the carbon and energy, whereas the glutamate probably served as a minor source of carbon and energy but supplied most of the nitrogen for growth.

Table 1 and Fig. 2 demonstrated that the mutants grew considerably better at pH 7.2 than at pH 6.0 on those substrates yielding abnormal growth. To confirm that this difference in growth was not due to defective uptake or metabolism of certain compounds the comparative capacity of mutant and wild cells to metabolize several of the substrates allowing abnormal growth of the mu-

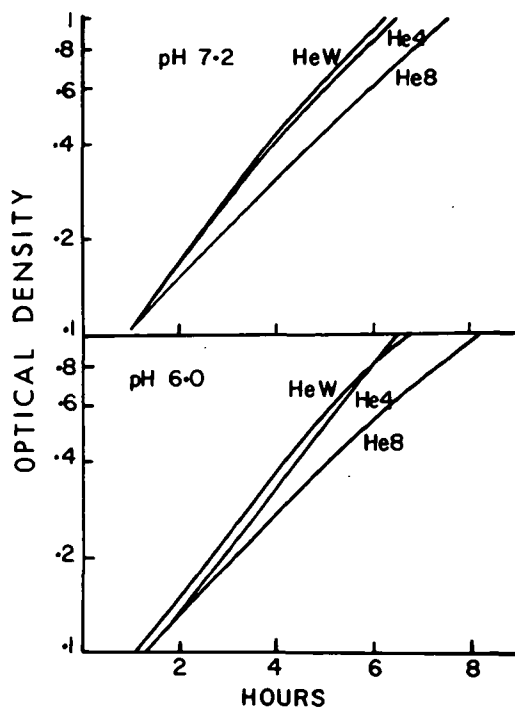


FIG. 1. Growth of wild-type *H. eutropha* (HeW) and mutant strains He4 and He8 in mineral-salts media containing 0.2% L-glutamate. Ordinate is a logarithmic scale. Growth was monitored by means of a Jouan biophotometer which records turbidity at 2-min intervals.

tants was examined manometrically (Table 2). The results demonstrated that pyruvate and alanine (one of the amino acids allowing expression of the mutation) were oxidized at comparable rates by both mutant and wild strains and, as seen with pyruvate, there was no relationship between growth rates, extracellular pH, and oxidation rates of the mutant strains. Oxidation rates for molecular hydrogen were also measured and revealed no significant differences between mutant and wild cells.

DISCUSSION

The large difference between the rates of reversion to prototrophy of the two mutant strains indicates that the two mutations occurred at different sites on the genome. The identical qualitative characteristics of He4 and He8 suggest that both mutations occurred within the same functional locus. This was further supported by the failure of the mutant strains to cross-feed one another.

The mutant isolation procedure was designed to obtain obligate heterotrophic mutants; however, since glutamate was employed as the or-

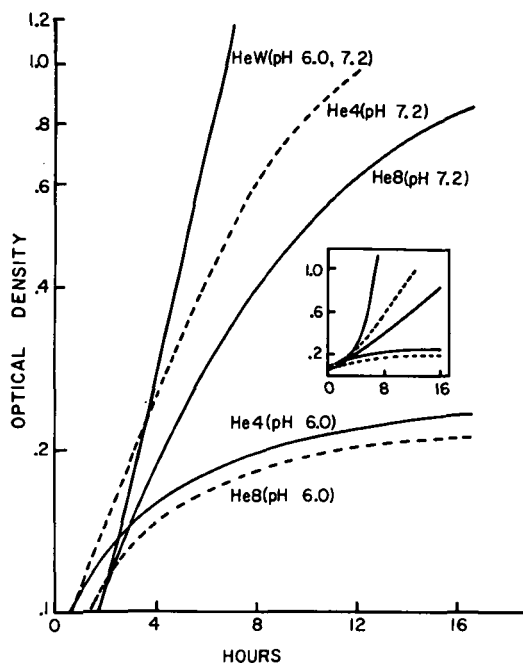


FIG. 2. Effect of pH on growth of wild-type *H. eutropha* (HeW) and mutant strains He4 and He8 in mineral-salts media containing 0.2% sodium pyruvate. Growth was monitored by means of a Jouan biophotometer. Ordinate is a logarithmic scale. Insert shows an arithmetic plot of the same data.

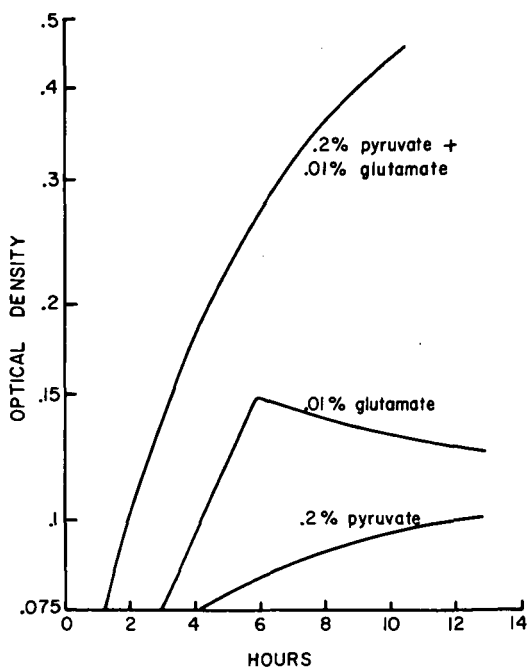


FIG. 3. Effect of a limiting concentration of glutamate on the growth of mutant strain He8 at pH 6.0. Media contained mineral-salts plus pyruvate, glutamate, or pyruvate and glutamate. Growth was monitored by means of a Jouan biophotometer. Ordinate is a logarithmic scale.

ganic substrate for those parts of the procedure requiring heterotrophic growth, it was also possible to select glutamate-requiring auxotrophs. For this reason other organic media (phenylalanine, fructose) were used to screen colonies growing on glutamate agar. The early observation that more than one amino acid could fulfill the nutritional needs of the mutants was not surprising due to the known metabolic relatedness of a number of amino acids. However, the fact that any one of many amino acids would allow He4 and He8 to grow normally, coupled with the inability of nonaminated compounds (including some keto analogues of amino acids) to support normal growth, strongly suggested that the requirement was for amino nitrogen. This was indicated further by the stimulation of mutant growth by low quantities of glutamate (Fig. 3). The ability of the mutants to oxidize normally those substrates producing abnormal growth eliminated the possibility that the mutation produced a general defect in the uptake or metabolism of nonaminated substrates. Since oxidation rates were similar for mutant and wild cells at either pH 6.0 or 7.2, extracellular pH was not adversely affecting the degradation of the tested substrates in mutant cells. In fact, oxida-

TABLE 2. Oxidation of pyruvate and L-alanine by wild-type (HeW) and mutant strains (He4, He8) of *H. eutropha*

Substrate ^a	pH	Oxygen uptake of strain ^b		
		HeW	He4	He8
Pyruvate	6.0	45.0	43.8	42.6
Pyruvate	7.2	26.9	25.3	26.3
Alanine	6.0	44.1	39.4	44.1

^a Warburg vessels contained 0.2% substrate in 0.04 M potassium phosphate buffer.

^b Oxygen uptake was measured as microliters per hour per milligram (dry weight) of cells. Values given are corrected for endogenous rates, which were approximately 30% of the activities in the presence of substrate.

tion rates at pH 6.0, where mutant growth was poor, were higher than at pH 7.2, where growth was better. It must therefore be assumed that the abnormal growth of the mutants, characterized by pH-dependent linear rates, is due to a deficiency in amino nitrogen synthesis and that this defect is influenced by extracellular pH. Since amino nitrogen synthesis in bacteria occurs primarily via reductive amination of carboxylic acid

to amino acid, the results suggest that the mutants are amination deficient.

ACKNOWLEDGMENT

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pH-Conditional, Ammonia Assimilation-Deficient Mutants of *Hydrogenomonas eutropha*: Evidence for the Nature of the Mutation¹

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Two amination-deficient mutants of *Hydrogenomonas eutropha*, characterized by pH-dependent linear growth on non-amino acid substrates, were investigated to determine the exact nature of the mutation. Glutamate dehydrogenase, the only aminating enzyme found in wild-type cells, was present at similar levels in mutant cells. Phenylalanine and aspartate, which allowed normal growth of the mutants, could transaminate 2-oxoglutarate to glutamate, whereas alanine, which does not support normal growth, could not transfer its amino nitrogen to form glutamate. In *H. eutropha*, L-alanine is apparently synthesized by β -decarboxylation of aspartate. Studies with NH_4^+ ions as the sole nitrogen source demonstrated that growth rates of the mutant strains were dependent on both extracellular pH and NH_4^+ ion concentration. Comparison of these results revealed that the growth rate of mutant cultures was proportional to the concentration of extracellular NH_3 . Wild-type cultures were not dependent on extracellular NH_3 since exponential growth rates did not vary with pH or NH_4^+ ion concentration. The results suggest that the mutant strains lack an NH_4^+ ion transport system and consequently are dependent on NH_3 diffusion which does not support optimal amination rates. The significance of the findings for the amino acid metabolism of *H. eutropha* is discussed.

Hydrogenomonas eutropha can utilize inorganic nitrogen as the sole nitrogen source for growth. Under such conditions biosynthesis and growth are dependent on reactions which incorporate ammonium nitrogen into organic molecules (amination). In the preceding paper (19) we described the isolation and growth characteristics of two amination-deficient mutants of *H. eutropha*. Both mutant strains grew normally on most amino acids, but demonstrated linear growth under conditions requiring amination (non-amino acid carbon and energy source and inorganic nitrogen supplied in the form of ammonium ions). Linear growth rates were dependent on extracellular pH, indicating that amination in the mutant strains was pH dependent.

Defective amination in the mutants could be due to any of several causes such as the production of an abnormal aminating enzyme, the defective synthesis of a normal aminating enzyme, or an intracellular concentration of intermediates in-

involved in amination that is below the level necessary for optimal enzymatic activity. An explanation involving any of these possibilities would have to include the effect of extracellular pH on amination in the mutant strains.

This paper describes studies which examine each of these possibilities and provides evidence that deficient amination in the *H. eutropha* mutants is due to a defect involving the permeation of ammonium nitrogen.

MATERIALS AND METHODS

See the preceding paper (19) for cultures, media, and growth conditions.

Preparation of cell-free extracts. Cultures were grown autotrophically or heterotrophically in shaken 1-liter flasks containing 500 ml of media. 1.5% Tryptic Soy Broth (Difco) or mineral-salts media containing 0.4% substrate were used for heterotrophic growth. Cells were harvested at log phase by centrifugation at 4 C and were suspended in potassium phosphate buffer (0.04 M, pH 7.2). The cell suspensions were disrupted by sonic treatment for 2 to 4 min in an ice bath by using a Biosonic II sonic oscillator (Bronwill Scientific Co., Rochester, N.Y.) at maximum power. The sonically treated suspensions were centrifuged at $20,000 \times g$ for 20 min at 4 C, and the supernatant fractions were dialyzed 18 to 24 hr against 4 liters of potassium phos-

¹ This report was taken from a dissertation submitted by L. F. S. to Catholic University in partial fulfillment of the requirements for the Ph.D. degree. It was presented in part at the 69th Annual Meeting of the American Society for Microbiology, Miami Beach, Fla., 4-9 May 1969.

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phate buffer (0.005 M, pH 7.2). The protein content of dialyzed cell-free extracts was determined by a standard biuret test (5). The dialyzed extracts were used for the enzyme assays.

Transaminase determinations. Reaction mixtures consisted of 30 μ moles of amino acid, 10 μ moles of appropriate keto acid, 0.2 ml of cell extract (2 mg of protein), and 20 μ g of pyridoxal phosphate in potassium phosphate buffer (0.04 M, pH 7.2) to a total volume of 1.0 ml. The mixtures were incubated with shaking at 30 C for 30 min to allow for equilibration of the transaminase reaction. Positive transaminase activity was demonstrated by detection and identification of the appropriate end-product amino acid by thin-layer chromatography by using ITLC type SG silica gel chromatography medium (Gelman Instrument Co., Ann Arbor, Mich.). The solvent system employed for separation of amino acids present in the reaction mixture was phenol-water (200:50, v/v). After development in this system and drying, chromatograms were sprayed with a solution of 0.5% ninhydrin in water-saturated butanol to reveal the presence and relative position of amino acids.

Glutamate and alanine dehydrogenase assays. Nicotinamide adenine dinucleotide (NAD)-specific glutamate dehydrogenase [L-glutamate:NAD oxidoreductase (deaminating), EC 1.4.1.2] and nicotinamide adenine dinucleotide phosphate (NADP)-specific glutamate dehydrogenase [L-glutamate:NADP oxidoreductase (deaminating), EC 1.4.1.4] were assayed spectrophotometrically at ambient temperature by the method of Strecker (18). Rates of NAD reduction and reduced NAD (NADH) or reduced NADP (NADPH) oxidation were measured at 340 nm by using a Hitachi Perkin-Elmer 139 spectrophotometer. Reductive amination was performed with the following mixture: 40 μ moles of 2-oxoglutarate, 40 μ moles of $(\text{NH}_4)_2\text{SO}_4$, 0.3 μ moles of NADH or NADPH, and 0.1 ml of cell extract (0.5 to 2.0 mg of protein) in potassium phosphate buffer (0.04 M at the desired pH) to a total volume of 3.0 ml. For oxidative deamination, 40 μ moles of L-glutamate and 0.3 μ moles of NAD were substituted for 2-oxoglutarate and NADH, respectively, and $(\text{NH}_4)_2\text{SO}_4$ was omitted. Alanine dehydrogenase [L-alanine:NAD oxidoreductase (deaminating) EC 1.4.1.1] was assayed in the same manner as reductive amination with glutamate dehydrogenase, except that 40 μ moles of pyruvate were used instead of 2-oxoglutarate.

Aspartate ammonia-lyase and alanine deamination assays. L-Aspartate ammonia-lyase (EC 4.3.1.1) activity was determined by the method of Vender and Rickenberg (20), which measures the rate of ammonia formation. Tubes contained 5.0 μ moles of L-aspartate, 5.0 μ moles of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, the amount of cell extract containing 0.5 to 2.0 mg of protein, and potassium phosphate buffer (0.04 M, pH 7.2) to a total volume of 2.0 ml. The mixture was incubated at ambient temperature for different time intervals after which the reaction was stopped by the addition of 0.4 ml of 5.0% trichloroacetic acid, and the ammonia produced was estimated colorimetrically by using Nessler's reagent by the procedure of Pelczar et al. (13). The deamination of alanine was followed by using a similar procedure except that whole cells [0.38 mg (dry weight)/ml] were incubated at 30 C with shaking in potassium phosphate buffer (pH 6.0) containing 0.2% L-alanine.

RESULTS

Glutamate dehydrogenase studies. Fincham isolated mutants of *Neurospora*, similar to the *H. eutropha* mutants in that they required amino acids as a source of nitrogen for normal growth, and found that they lacked the aminating enzyme glutamate dehydrogenase (NADP-linked; reference 4). We therefore investigated the possibility that deficient amination in the *H. eutropha* mutants was due to decreased activity of the enzyme responsible for catalyzing the incorporation of ammonia into amino acids. In bacteria there are only three well-known enzymes capable of performing this function: L-glutamate dehydrogenase, L-alanine dehydrogenase, and L-aspartate ammonia-lyase (11). These enzymes catalyze the incorporation of ammonium nitrogen into 2-oxoglutarate, pyruvate, and fumarate, forming glutamate, alanine, and aspartate, respectively. Since it was not known which of these catalysts functioned in *H. eutropha*, extracts of wild-type cells (HeW) grown on amino acid and non-amino acid substrates were assayed for each of the three enzymes. The results are shown in Table 1. Glutamate dehydrogenase was the only aminating enzyme found in wild-type cells, being present in all extracts tested. Extracts of mutant strains He4 and He8 grown on Tryptic Soy Broth were then tested for glutamate dehydrogenase and were found to possess enzyme levels comparable to

TABLE 1. Examination of *H. eutropha* cell extracts for enzymes capable of assimilating ammonium ions into organic acids

Enzyme	Growth substrate ^a	pH ^b	Coenzyme	Specific activity ^c
Glutamate dehydrogenase	TSB	7.2	NADH	9.5
	TSB	7.2	NADPH	40.5
	TSB	6.0	NADPH	0
	TSB	8.0	NADPH	65.7
	TSB	7.2	NADP	10.0
	Glutamate	7.2	NADPH	81.0
	Fructose	7.2	NADPH	17.6
	H ₂ , O ₂ , CO ₂	7.2	NADPH	22.8
	Pyruvate	7.2	NADPH	26.2
Alanine dehydrogenase	TSB	7.2	NADH	0
	TSB	7.2	NADPH	0
	Fructose	7.2	NADH	0
	Fructose	7.2	NADPH	0
	Pyruvate	7.2	NADH	0
Aspartate ammonia-lyase	TSB	7.2		0
	Glutamate	7.2		0
	Pyruvate	7.2		0

^a Refers to the substrate that cells were grown on for preparation of extract. TSB, tryptic soy broth.

^b Refers to the pH of the enzyme assay mixture.

^c Nanomoles of coenzyme oxidized (or reduced in one case) per milligram of protein per minute. Activities are corrected for NADH- and NADPH-oxidase-specific activities which ranged from 0 to 14.

those present in wild-type cells (relative activities at pH 7.2 compared to HeW were 1.04 for He4 and 1.00 for He8). Thus glutamate dehydrogenase synthesis and activity are apparently normal in the mutant cells.

Other characteristics of the enzyme were studied by using wild-type cells (Table 1). In extracts derived from cells grown on Tryptic Soy Broth, glutamate dehydrogenase activity was found with both NADH and NADPH. At pH 7.2, enzyme activity with NADPH as cofactor was over four times greater than with NADH. Since the reductive reaction with NADPH is generally biosynthetic (6, 9, 20) further studies of the enzyme were performed by using this cofactor. No activity was found at pH 6.0, but as the pH was increased to 8.0, enzyme activity became greater, as is characteristic of glutamate dehydrogenase. When NADP-linked glutamate dehydrogenase activity was measured in the direction of glutamate degradation, activity was 25% of that found in the direction of glutamate synthesis. Although NADP-linked glutamate dehydrogenase was found in all extracts tested, the specific activity of the enzyme varied considerably. Extracts derived from amino acid-grown cells contained much greater enzyme levels than those derived from various non-amino acid substrates. For example, glutamate extracts were approximately three to four times more active than fructose, pyruvate, or autotrophic extracts. Tryptic Soy Broth, rich in various sources of amino acids, yielded extracts which had only 50% the specific activity obtained with glutamate extracts, but still contained almost twice the activity found in extracts derived from cells grown on non-amino acid substrates. Except for determining the effect of pH on enzyme activity, assays were performed at pH 7.2 since we assumed that the intracellular pH at which glutamate dehydrogenase normally functions is near neutrality.

Effect of NH_4^+ ion concentration on growth of mutant cultures. Since the aminating enzyme in mutant cells was apparently normal, we examined the possibility that the intracellular ammonium nitrogen level of mutant cells was below that necessary for optimal amination. Since NH_4^+ ions were the source of inorganic nitrogen used in the growth studies, this situation could occur if the normal mechanism were impaired, involving either NH_4^+ ion permeation or the maintenance of an optimal intracellular concentration of NH_4^+ ions. To test these possibilities, we increased the extracellular NH_4^+ ion concentration above the normal level of 15 mM to determine whether this would affect the growth of mutant cells under aminating conditions. Growth

rates of mutant strain He8 on pyruvate at pH 6.0 eventually became linear at each NH_4^+ concentration but linear growth rates increased drastically as the extracellular NH_4^+ concentration was raised from 15 to 150 mM (Fig. 1). In contrast to the mutant culture, growth of HeW was identical and exponential within this range of NH_4^+ ion concentrations. Further stimulation of mutant growth using higher concentrations of NH_4^+ was not observed since $(\text{NH}_4)_2\text{SO}_4$ became inhibitory to the growth of both mutant and wild-type cells at concentrations above 150 mM.

Effect of pH on growth of mutant cultures. The stimulation of mutant growth by increased levels of ammonium ions suggested that a defect in the permeation of this nitrogen source was responsible for deficient amination. But this effect alone would not explain why mutant growth improved when the extracellular pH was increased from 6.0 to 7.2 (19). HeW and He8 were grown in pyruvate media containing constant NH_4^+ ion concentrations but differing widely in pH (Fig. 2) to define the pH effect more precisely. Except for initial periods of logarithmic growth, growth rates of mutant cells were linear, yielding a constant increase in optical density (OD) per time interval. When the extracellular pH was increased at intervals between pH 6.0 and 8.0, linear growth rates of mutant He8 became greater. For example, at pH 6.0 and 7.1, the constant linear rates were 0.005 and 0.056 OD units/hr, respectively. Thus the mutant growth rate increased 11-fold when the pH was increased from 6.0 to 7.1. Growth curves with wild-type *H. eutropha* were exponential and essentially identical over the entire pH range tested.

Dependence of mutant growth on extracellular NH_3 . Since mutant growth rates were affected by either pH or NH_4^+ concentration, deficient amination could not be explained solely in terms of defective permeation of NH_4^+ ions. The fact that linear mutant growth rates increased both with increasing pH and NH_4^+ ion concentration suggested that some common factor determined both by pH and NH_4^+ ion concentration was responsible for controlling the growth of mutant cells. The factor which fits this description is NH_3 , since it is this substance that increases with pH when NH_4^+ concentration is constant, and increases with NH_4^+ concentration when pH is constant, according to the Henderson-Hasselbalch equation for the dissociation of weak acids. Inspection of our growth data revealed that similar mutant growth patterns were obtained for similar NH_3 concentrations derived from widely different combinations of pH and NH_4^+ concentrations. In addition, linear growth rates of mutant cells were proportional to the NH_3 concen-

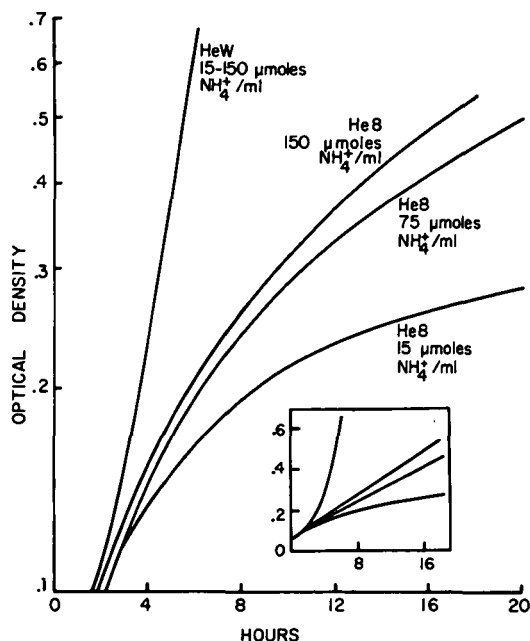


FIG. 1. Effect of NH_4^+ concentration on growth of wild-type *H. eutropha* (HeW) and mutant strain He8 in mineral-salts media (pH 6.0) containing 0.2% sodium pyruvate. Growth was monitored by means of a Jouan biophotometer which records turbidity at 2-min intervals. Ordinate is a logarithmic scale. Insert shows an arithmetic plot of the same data.

tration calculated from different combinations of extracellular pH and NH_4^+ concentrations (Fig. 3). Growth of wild-type cells under aminating conditions was indifferent to wide variations in extracellular pH, NH_4^+ concentration, or the different concentrations of NH_3 produced under these conditions, since exponential growth rates of HeW remained essentially constant.

Effect of extracellular NH_3 on pyruvate oxidation rates. Wiley (Ph.D. Thesis, Washington State University, Pullman, 1965) studied the effect of extracellular NH_3 (as related to pH and NH_4^+ concentration) on the metabolism of *Bacillus pasteurii*. He suggested that diffusion of NH_3 was necessary for the transport of nutrients across the cell membrane to explain why this organism requires an alkaline pH for growth. We therefore measured the effect of extracellular NH_3 on the utilization of non-amino acids by mutant and wild type *H. eutropha* (Table 2). The oxidation of pyruvate was measured in the presence and absence of conditions which produced relatively high extracellular concentrations of NH_3 (pH 8.0, with and without 45 mM NH_4^+). Under these conditions, a 1.8 mM concentration of extracellular NH_3 is theoretically produced.

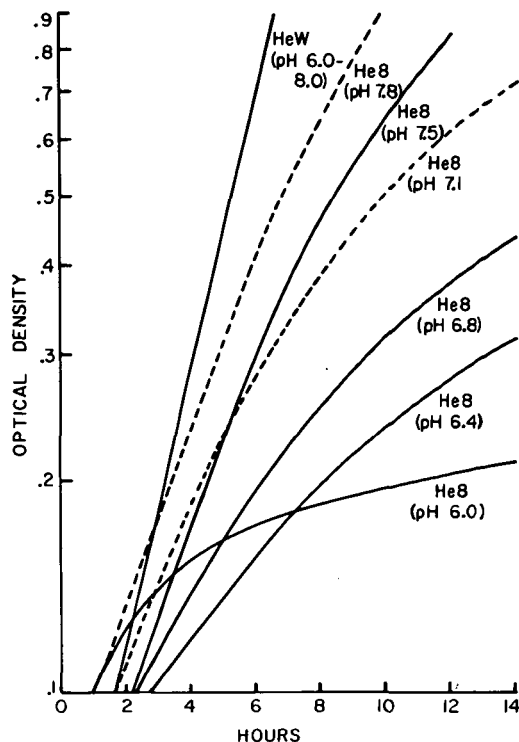


FIG. 2. Effect of pH on growth of wild-type *H. eutropha* (HeW) and mutant strain He8 in mineral-salts media containing 0.2% sodium pyruvate. Growth was monitored by means of a Jouan biophotometer. Ordinate is a logarithmic scale.

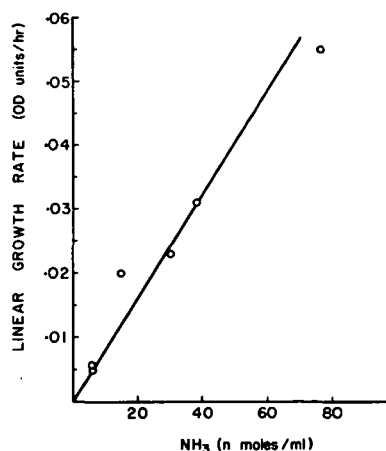


FIG. 3. Relationship between extracellular ammonia concentration and linear growth rates of *H. eutropha* mutants.

However, no difference was observed between the oxidation rates of mutant and wild strains under similar conditions. Although pyruvate oxidation

rates were increased by approximately 45% in the presence of NH_4^+ ions (and NH_3), this effect was shared equally by mutant and wild-type cells.

Growth with other sources of inorganic nitrogen. Since *H. eutropha* is capable of utilizing various inorganic nitrogen sources other than the ammonium ion (14), growth of mutant strains He4 and He8 was attempted in pyruvate media containing urea or KNO_3 . Growth of the mutants was not detectable with either of these nitrogen sources at any pH from 6.0 to 8.0, the same pH range at which NH_3 -dependent growth was observed. In contrast, HeW grew exponentially with both nitrogen sources throughout the same pH range.

Growth on alanine and alanine metabolism. In the previous paper (19) we demonstrated that most amino acids were capable of functioning as amino nitrogen sources, and therefore allowed normal growth of He4 and He8. Exceptions to this were alanine and three other amino acids which did not support normal growth, presumably because they could not be utilized by *H. eutropha* as sources of amino nitrogen. This would indicate that reductive amination is required for growth on these amino acids. This is supported by studies with alanine. When alanine was supplied as the sole carbon, energy, and nitrogen source for growth, wild-type cells grew exponentially in the pH range 6.0 to 8.0, but growth of the mutant cultures did not occur at any pH value. The two mutant strains as well as HeW deaminated alanine and caused extracellular accumulations of NH_4^+ at similar rates. After 3 hr approximately 2.5 μmoles of NH_4^+ /ml were produced which at pH 6.0 yields 1.0 nmole of NH_3

per ml. Although HeW can utilize this level of NH_4^+ ions for growth, this concentration of NH_3 is adequate for only meager growth of the mutants as seen in Fig. 3. When NH_4^+ ions were added along with alanine, growth curves of mutant strain He8 were very similar to those seen in Fig. 2 with pyruvate. As with pyruvate, growth of wild-type cells was exponential, but growth rates of mutant cells were linear and pH dependent. Although allowing pH-dependent growth, the presence of NH_4^+ ions did not affect the oxidation of alanine. Rates of oxidation in the presence of NH_4^+ were similar for mutant and wild cells and compared almost exactly with oxidation rates in the absence of NH_4^+ ions.

Transaminase studies. Since the end product of reductive amination in *H. eutropha* is apparently glutamate, this amino acid probably acts as primary amino donor in the biosynthesis of other amino acids. Consequently, the ability of an amino acid to act as a source of amino nitrogen for growth, in effect bypassing the need for reductive amination, would be related to the ability of the cell to utilize that amino acid for glutamate synthesis, either by degradation or transamination. The mutant growth studies predict that in *H. eutropha* most amino acids can function in this manner, whereas four, including alanine, probably cannot. Of the amino acids used in the growth studies, the only ones normally degradable to glutamate by bacteria are proline and histidine. As expected, both of these amino acids support normal growth of the mutant cultures. Whether the remaining amino acids could supply amino nitrogen to glutamate would depend on the presence or absence of transamination to glutamate. We therefore examined the transaminase activity of HeW with regard to a few of the amino acids not normally degradable to glutamate. In agreement with the growth studies, we could not detect an alanine \rightleftharpoons glutamate transaminase (L-alanine:2-oxoglutarate aminotransferase, EC 2.6.1.2) in cell extracts of *H. eutropha*. In contrast, phenylalanine and aspartate, two of the amino acids which support normal growth of the mutants, transferred their amino groups to form glutamate in the presence of HeW cell extract.

The absence of a glutamate \rightleftharpoons alanine transaminase raised the question of how alanine is synthesized by *H. eutropha*. This was apparently answered during the aspartate to glutamate transamination experiments. Incubation of HeW cell extract with aspartate always yielded a chromatographic spot that reacted with ninhydrin, was distinguishable from β -alanine and was identified as α -alanine. This compound appeared whether 2-oxoglutarate was present or absent, and sug-

TABLE 2. Effect of extracellular ammonia and ammonium ions on the oxidation of pyruvate by wild-type (HeW) and mutant strains (He4, He8) of *H. eutropha*^a

Strain	Pyruvate concn (%)	O ₂ uptake ^b	
		With NH_4^+ ^c	Without NH_4^+
HeW	0.2	51.9	33.8
He4	0.2	46.6	34.7
He8	0.2	52.5	34.7
HeW	0.4	55.3	36.0
He4	0.4	52.5	38.8
He8	0.4	59.7	41.9

^a Warburg vessels contained sodium pyruvate in 0.04 M potassium phosphate buffer (pH 8.0).

^b Microliters per hour per milligram (dry weight) of cells. Values given are corrected for endogenous rates.

^c Vessels contained 45 μmoles of $(\text{NH}_4)_2\text{SO}_4$ per ml yielding 1.8 μmoles of NH_3 per ml at pH 8.0.

gested that α -alanine synthesis in *H. eutropha* occurs via aspartate 4-decarboxylase (L-aspartate 4-carboxylase, EC 4.1.1.12).

DISCUSSION

Several lines of evidence demonstrate that the two mutant strains of *H. eutropha* are unable to assimilate inorganic nitrogen normally; therefore growth (and presumably glutamate synthesis) on nonaminated substrates is dependent on the NH_4^+ concentration. Phenylalanine and aspartate, which support normal growth of the mutants, could transaminate 2-oxoglutarate to glutamate, whereas alanine, which does not support normal growth, cannot donate its amino group to form glutamate. Thus growth of the mutants on alanine would require amination and would be restricted.

Since He4 and He8 contained normal levels of glutamate dehydrogenase and this was the only aminating enzyme found in *H. eutropha*, defective amination could not be explained in terms of a defective enzyme. This is especially true since our comparison of mutant and wild-type enzyme levels were performed by using NADP as the cofactor for glutamate dehydrogenase, and Joseph and Wixom (8) reported recently that an NADP-specific glutamate dehydrogenase is responsible for ammonia incorporation in *H. eutropha*.

Deficient amination could also occur if one of the substrates for the amination reaction (2-oxoglutarate or ammonia) were present in limiting amounts. It is unlikely that 2-oxoglutarate was limiting because *H. eutropha* is known to possess an active Krebs cycle (17) and the mutants are able to oxidize substrates such as pyruvate at normal rates. Also, if 2-oxoglutarate were limited, the mutants would be expected to grow normally when supplied with exogenous 2-oxoglutarate; however, as reported previously (19), growth on 2-oxoglutarate and other Krebs intermediates was abnormal.

The fact that mutant growth rates increased with increasing NH_4^+ concentration while growth of HeW was independent of NH_4^+ suggested that the mutants might have a defective NH_4^+ uptake mechanism, and growth was therefore dependent on a high concentration gradient forcing NH_4^+ into the cells. However, since increasing the pH also increased mutant growth rates (at constant levels of NH_4^+), an explanation taking both NH_4^+ concentration and pH into account was required.

The extracellular concentration of NH_3 is determined by the NH_4^+ concentration and the pH of the medium. The formation of extracellular

NH_3 as related to pH and NH_4^+ concentration, and its free diffusion into the bacterial cell has been demonstrated by Zarlengo and Abrams (22) with cell suspensions of *Streptococcus faecalis*. Also, the free diffusion of NH_3 into various plant and animal cells (1, 7) and other biological systems (15) has been reported. However, since *S. faecalis*, as well as animal cells, requires a number of amino acids, which can also supply nitrogen for growth, it could not be determined whether the amount of NH_3 entering these cells by diffusion would be sufficient as the sole source of nitrogen. It was seen in Fig. 3 that linear mutant growth rates were proportional to the extracellular concentration of NH_3 . Also, since there is always much more NH_4^+ ion than NH_3 present at near neutral pH the level of NH_3 probably remained fairly constant but limiting during the initial logarithmic periods of mutant growth. The gradual decrease in growth rates to constant linear levels could occur if the rate of conversion of NH_4^+ to NH_3 fell below NH_3 uptake rates by the increasing cell population, but whether this or some other explanation accounts for the dependence of mutant growth rates on NH_3 concentration has not been determined. Since growth of HeW is not dependent on the extracellular NH_3 concentration, we might conclude that the wild-type organism has an NH_4^+ ion permeation mechanism and that the mutants have lost this mechanism and must rely on concentration-dependent diffusion of NH_3 for growth.

Mutants similar to these, but involving potassium permeability, have been obtained from *Escherichia coli* (3, 10, 16) and *S. faecalis* (23). From both species two types of potassium-deficient mutants have been isolated. One type is defective in K^+ uptake, whereas the other is normal for K^+ uptake but unable to accumulate and retain K^+ in the cell. Mutants defective in K^+ retention have also been described for *Bacillus subtilis* (21). The K^+ uptake and K^+ retention mutant types both demonstrate abnormal growth at rates dependent on extracellular K^+ concentration. Therefore they are indistinguishable on the basis of growth studies, and must be subjected to K^+ uptake and K^+ retention studies. Based on the results of our growth studies the *H. eutropha* mutants could be either NH_4^+ -uptake or NH_4^+ -retention negative. The main difference between our system and that of the K^+ -deficient mutants is the possibility of the entrance or exit of NH_4^+ ions via pH-dependent dissociation to NH_3 . Also since growth rates of the *H. eutropha* mutants are proportional to extracellular NH_3 , this would indicate that the NH_4^+ uptake or retention system is nonfunctional, rather than deficient as in the case of the K^+ mutants.

We are unable to experimentally determine whether He4 and He8 are NH_4^+ -uptake or NH_4^+ -retention negative because the sensitivity of the Nessler's test is inadequate. Uptake or leakage of potassium from mutants was determined by using radioactive ions, but this procedure is not readily available with the NH_4^+ ion.

The growth studies involving NO_3^- , urea, and alanine as nitrogen sources suggest that the mutants are retention-negative. It is unlikely that both NO_3^- and urea permeate into the cell by means of the same mechanism as NH_4^+ , so the absence of mutant growth with these nitrogen sources suggests that they may be taken into the cell and converted to NH_4^+ , which then leaks out of the cell before reaching usable levels. When the mutants were supplied with alanine as the sole carbon and nitrogen source, extracellular NH_4^+ did accumulate due to the deamination of alanine by the mutants. This indicates that upon deamination the ammonium nitrogen escapes from the cell and does not build up to intracellular levels necessary for amination. In addition, ammonium nitrogen never reached an extracellular level high enough for NH_3 -dependent growth to occur by diffusion.

To our knowledge, the amination-deficient mutants of *H. eutropha* are the first organisms reported to be dependent on NH_3 diffusion for their nitrogen supply. Since this condition is clearly inadequate under circumstances requiring amination, and especially at low pH, it is quite likely that those organisms capable of assimilating nitrogen into amino acids must rely primarily on transport mechanisms for the uptake of inorganic nitrogenous ions.

Our data suggest that *H. eutropha* synthesizes α -alanine by the β -decarboxylation of aspartate. Since this seems to be an uncommon reaction which has also been reported in *Alcaligenes faecalis* (12), one can speculate on the taxonomic significance of this similarity in light of the recent suggestion by Davis et al. (2) that *H. eutropha* be placed in the genus *Alcaligenes*.

ACKNOWLEDGMENT

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Bicarbonate Requirement for Elimination of the Lag Period of *Hydrogenomonas eutropha*

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Carbon dioxide and oxygen concentrations have a profound effect on the lag period of chemoautotrophically grown *Hydrogenomonas eutropha*. Minimum lag periods and high growth rates were obtained in shaken flask cultures with a prepared gas mixture containing 70% H₂, 20% O₂, and 10% CO₂. However, excessively long lag periods resulted when the same gas mixture was sparged through the culture. The lag period was shortened in sparged cultures by decreasing both the pO₂ and the pCO₂, indicating that gas medium equilibration had not occurred in shaken cultures. The lag period was completely eliminated at certain concentrations of O₂ and CO₂. The optimum pO₂ was 0.05 atm, but the optimum pCO₂ varied according to the pH of the medium and physiological age of the inoculum. At pH 6.4, the pCO₂ required to obtain immediate growth of exponential, postexponential, and stationary phase inocula at equal specific rates was 0.02, 0.05, and 0.16 atm, respectively. With each 0.3-unit increase in the pH of the medium, a 50% decrease in the CO₂ concentration was needed to permit growth to occur at the same rate. The pCO₂ changes required to compensate for the pH changes of the medium had the net effect of maintaining a constant bicarbonate ion concentration. Initial growth of *H. eutropha* was therefore indirectly related to pCO₂ and directly dependent upon a constant bicarbonate ion concentration.

Species of *Hydrogenomonas* are partially characterized by their ability to grow in certain ranges of oxygen concentration (*Bergey's Manual*, 7th ed.; 3, 4), the extent of growth being the primary determinant. Critical data on the effect of the partial pressure of oxygen on exponential growth rates have been presented by Schlegel and Lafferty (9) and Bongers (1). Although CO₂ is a primary substrate for autotrophic growth, there are little data on the effect of CO₂ concentration on cell growth. Until recently (1), only the effects of gross variations in pCO₂ were reported. The idea that cell growth would respond to small changes in CO₂ concentration seems to have been neglected.

It is tacitly assumed that a nutritionally complete medium which supports exponential growth of cells also provides the most favorable environment for initial growth of an inoculum, even if the inoculum consists of physiologically old cells. Data presented in this paper with *Hydrogenomonas eutropha* show that inocula from cultures of different physiological ages have different initial growth requirements for CO₂. When the appropriate CO₂ concentration was provided, exponential growth began immediately at max-

imum specific rates. The optimum pCO₂ for immediate growth varied with the pH of the medium. These data showed that bicarbonate was the species of CO₂ used by the cells. Need for the unique pCO₂ changed after one to two cell generations, and thereafter all cultures had a common CO₂ requirement.

MATERIALS AND METHODS

H. eutropha inocula were prepared as liquid cultures grown chemoautotrophically at 31°C on a rotary shaker in 500-ml baffled flasks containing 100 ml of salts medium (6, 7). The premixed gas atmosphere contained 70% hydrogen, 20% oxygen, and 10% carbon dioxide. After incubation, a special rubber stopper (7), by which the flask was connected to the gas manifold, was replaced with a sterile cotton plug. Stock cultures could be stored for a month at room temperature without significant loss of viability. Culture purity was determined by microscopic examination and by plating on Trypticase soy agar. Viable cell counts determined on Trypticase soy agar in air or on mineral salts medium (Noble agar base) in the gas mixture were the same; reliable cell counts therefore could be made under heterotrophic conditions (11).

In experiments in which growth was studied as a function of the partial pressures of oxygen and carbon

dioxide, a 2.5-liter fermentor containing 1 liter of medium was used. The fermentor consisted of a cylindrical glass vessel containing individual spargers for H_2 , O_2 , and CO_2 . Near the base of the vessel was a port fitted with a serum bottle stopper through which a combination pH electrode was inserted. Culture samples were also withdrawn by syringe through the serum bottle stopper. Gas dispersion was accomplished by a double-bladed impeller driven by a "Lightnin" (Fisher Scientific Co., Fair Lawn, N.J.) air-driven motor mounted on the metal head plate. The impeller bearing in the head plate was a Teflon sleeve with an "O" ring to provide a positive seal (Chesapeake Stirrer, Van Dyk Research Corp., Hackettstown, N.J.). The head plate was also fitted with an addition port and a gas exhaust line.

Gas proportions were calculated from relative gas flow rates which were regulated by individual flow meters. Each gas entering the fermentor was filtered through sterile glass wool; excess gas was vented through the head plate and a filter before being discharged into an air exhaust duct where dilution removed the explosive hazard. Equilibration between the gas and liquid phases was established by preflushing the medium with the gases for 30 min prior to inoculation. With few exceptions the minor gas component in a given mixture had a flow rate that exceeded 100 ml per min.

Growth was measured by the increase in optical density (OD) at 660 nm (Gilford spectrophotometer, Gilford Instrument Laboratories, Inc., Oberlin, Ohio) in a cell of 1-cm light path by using uninoculated gas equilibrated medium as reference. OD increases in short-term growth experiments were directly proportional to viable counts. The viable count calculated for an OD of 1 was 2.8×10^8 cells per ml in these cultures as well as in cultures grown to higher densities. Viable count, OD, and dry weight were proportional to an OD of 0.400. Cultures having higher densities were diluted to the proportional range, and the OD was calculated by using the dilution factor. The volume of the inoculum was adjusted as necessary to provide approximately the same initial cell concentration (5×10^7 cells per ml) in the fermentor in all experiments.

RESULTS

The composition of the gas atmosphere for growing *H. eutropha* autotrophically in shaken cultures had been chosen to provide the highest growth rate (6, 7). Under these conditions a 2- to 4-hr lag period was always observed prior to exponential growth when a 1% inoculum from postexponential phase or stationary phase cultures was used (Fig. 1). A slight lag period occurred when the inoculum was taken from an exponentially growing culture. The rate of exponential growth in all cultures was the same regardless of the type of inoculum used.

Reproducible growth responses described above were obtained with inocula from fresh cultures. Other experiments performed with stored inocula (stationary phase) were of incidental in-

terest because they suggested effective methods of storing stock cultures. It was found that inocula changed within 3 days when stored at room temperature in flasks sealed with rubber stoppers, whereas inocula stored for a week in flasks with fitted cotton plugs grew like the fresh inoculum on subculture.

A simple experiment (Table 1) clearly illustrated that in some way aerobic conditions during storage were required to maintain cell viability and to prevent lysis. Samples of a culture were distributed as shown to provide different degrees of accessibility to air. When a large surface-to-volume ratio existed (lines 2 and 5), viable counts and OD did not change during 30 days storage. The viable count decreased 100-fold and lysis accounted for a 50% decrease in turbidity when a small surface-to-volume ratio existed. It was immaterial whether the tube was

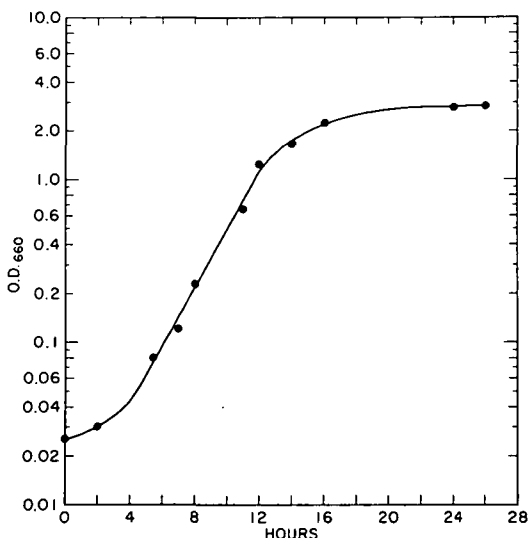


FIG. 1. Growth curve of *Hydrogenomonas eutropha* cultured chemoautotrophically (100 ml) at 31°C on a gyratory shaker. The gas mixture contained 70% H_2 , 20% O_2 , and 10% CO_2 .

TABLE 1. Effect of storage conditions on *Hydrogenomonas eutropha*

Storage conditions ^a	pH	OD ₆₆₀	Viable count/ml
None (original culture)	6.4	2.260	6.0×10^8
Flask (50-ml) cotton plug (10 ml) ^b	6.4	2.090	6.5×10^8
Test tube, screw cap (10 ml)	6.3	1.220	3.3×10^7
Test tube, plastic cap (10 ml)	6.4	1.170	6.7×10^7
Test tube, plastic cap (1.5 ml)	6.6	2.200	7.5×10^8

^a 30 days at room temperature.

^b Sample volume.

sealed (line 3) or had access to air (line 4). Even when cell viability was unchanged during storage, some subtle change occurred in the cells because on subculture the lag period extended to about 6 hr. Inocula used in the experiments described below were obtained from fresh cultures or from cultures stored aerobically for less than 1 week.

Effect of pO_2 and pCO_2 on initial growth. Larger volume cultures were grown in a 2.5-liter fermentor where adequate gas diffusion required sparged gases distributed by an impeller. Individual flow meters permitted accurate control and rapid modification of the gas mixture. Cultures grown in the standard medium with the usual gas mixture (70% H_2 , 20% O_2 , and 10% CO_2) had 18- to 24-hr lag periods. The lag periods became shorter as the partial pressures of O_2 and CO_2 were decreased to 0.05 atm, indicating excess oxygen, carbon dioxide, or both, were significant factors in the lag period.

The optimum pO_2 and pCO_2 for a minimum lag period were determined with three partial pressures of O_2 and CO_2 (0.025, 0.05, and 0.09 atm). In these experiments the gas mixture consisted of the indicated partial pressures of O_2 and CO_2 ; the remaining gas in the mixture was hydrogen. Although the pH_2 varied between experiments, it was in excess of 0.50 atm and was not limiting (1, 8). Because CO_2 altered the pH of the medium, the pH was adjusted during the equilibration period and was monitored during the experiment. During these short periods of growth, the adjusted pH remained constant.

Figures 2A, B, and C show that at pH 6.4 the inoculum grew without a lag period with most of the gas combinations tested, and the pO_2 and pCO_2 were much lower than was required for shake cultures. Most rapid exponential growth occurred with a pCO_2 of 0.05 atm and a pO_2 of either 0.05 or 0.09 atm. The optimum CO_2 concentration was easily defined, since the growth rates fell off sharply on either side of 0.05 atm. The optimum pO_2 range was not as narrow. A higher rate of growth was obtained with 0.09 atm of O_2 ($\mu = 0.33$), but the longer sustained growth was obtained with 0.05 atm of O_2 ($\mu = 0.24$; Fig. 2B), and this concentration of O_2 was considered optimal. With 0.05 atm of O_2 and CO_2 , a break in the growth rate consistently occurred after about 3 hr; several hours thereafter, growth again resumed at the original rate. The break could be circumvented and the initial rate of growth could be maintained without interruption by adjusting the pCO_2 to 0.02 atm before the change in rate. Growth then continued at the same specific rate to OD of 10 if additional adjustments were made as the CO_2 and O_2 became limiting (*unpublished results*).

The growth rate was depressed and approximated that shown in Fig. 2B for 0.09 atm of CO_2 when the pH of the medium was increased only 0.31 unit to pH 6.71. In another series of experiments conducted at pH 6.71 with various partial pressures of CO_2 , it was found that a pCO_2 of 0.025 atm (Fig. 2D) was needed to duplicate the former growth rate ($\mu = 0.24$). When the pH of the medium was increased again by 0.32 units to pH 7.03, the pCO_2 required to achieve the same growth rate decreased again by half to 0.012 atm; both 0.05 and 0.025 atm CO_2 were inhibitory at this pH . In all of these experiments, the optimum pO_2 continued to be 0.05 atm.

For each incremental increase in pH of approximately 0.3 unit, it was found that a 50% decrease in pCO_2 was needed to obtain an equivalent growth rate; consequently, with an overall pH increase of only 0.6 unit, a fourfold reduction in pCO_2 was required. The pH change per se had no apparent effect on growth, since the same growth rates were achieved at each pH tested (Fig. 2D). These results are explained on the basis of the CO_2 -bicarbonate ion equilibrium. At constant pH , the bicarbonate ion concentration is directly proportional to the pCO_2 . With a constant pCO_2 , the bicarbonate ion concentration in equilibrium with CO_2 doubles with each 0.3 pH unit increase (10). The empirically determined pCO_2 required to attain a given specific growth rate at the pH levels tested was the same as the calculated pCO_2 required to maintain a constant bicarbonate concentration of $1.6 \times 10^{-3} M$ (10). Growth of the inoculum was therefore dependent upon a constant bicarbonate ion concentration and independent of the absolute pCO_2 . Bicarbonate ion, not free CO_2 , was critical in the first hours of growth of *H. eutropha*.

Effect of physiological age of the inoculum on the CO_2 requirement. Chesney (2) showed that the lag period observed on subculture was related to the physiological age of the inoculum. Cultures used as inocula in the previous experiments were harvested in the postexponential phase of growth (OD₆₆₀ of 1.3 to 1.7; Fig. 1). Consequently, it was of interest to determine the effect of pO_2 and pCO_2 on initial growth with exponential phase cells (OD₆₆₀ of 0.700) and with stationary phase cells (OD₆₆₀ of 2.0 to 3.5). It is also known that the size of the inoculum influences the length of the lag period (5). This variable was controlled by adjusting the volume of the inoculum to provide the same final cell concentration throughout.

When exponential phase inocula were grown at pH 6.45 with 0.05 atm CO_2 , the growth rate was very low, contrary to what was described above

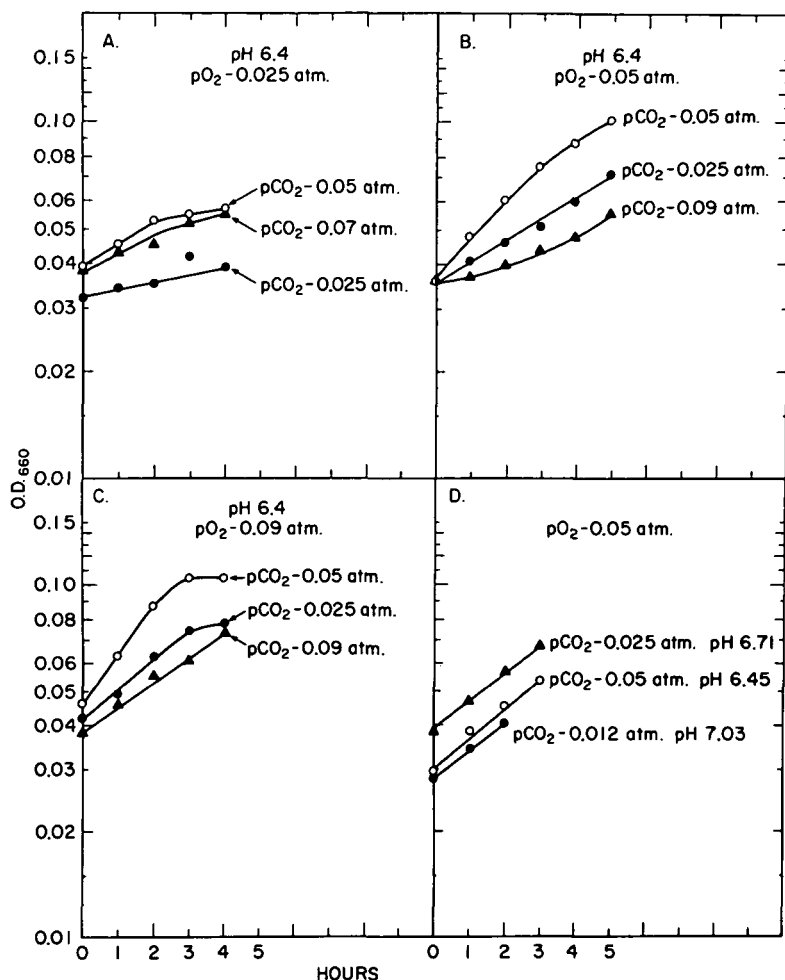


FIG. 2. Initial growth response of *Hydrogenomonas eutropha* as a function of pO_2 and pCO_2 at 24°C. Gases were sparged through the cultures which were grown in a fermentor containing 1 liter of medium. The pH adjusted during gas equilibration remained stable during the experiment. A 2% postexponential inoculum having an OD_{660} of 1.6 was used.

with postexponential phase inocula. As shown in Fig. 3, the desired rate of growth ($\mu = 0.24$) was obtained when the pCO_2 was reduced 2.5-fold to 0.02 atm (6.6×10^{-4} M bicarbonate at equilibrium). Although the optimum pCO_2 was lower for exponential phase inocula, a similar adverse growth rate response occurred with half or twice the CO_2 concentration. The effect of increasing the pH to 6.71, while holding the CO_2 constant at 0.02 atm, is shown in Fig. 3. Growth was inhibited as a result of doubling the bicarbonate concentration. When the pCO_2 was reduced to 0.01 atm (not shown), growth occurred at the expected rate ($\mu = 0.24$). It is equally valid to consider these results from the converse point of view; namely, at pH 6.71, 0.02 atm CO_2 provided excess bicarbonate, but by reducing the pH

to 6.45 the bicarbonate concentration was decreased to the appropriate concentration.

Stationary phase inocula have the same pattern of growth responses to CO_2 as found with the other types of inocula. Quantitatively, stationary phase inocula required a much higher pCO_2 to begin immediate exponential growth at a specific growth rate of 0.24; 0.16 atm CO_2 (5.3×10^{-3} M bicarbonate at equilibrium) was optimal. The relationship between optimum pCO_2 and the pH of the medium with inocula from older cultures is shown in Fig. 4 at pH 6.40, 6.70, and 6.90. Note that since the pH increase from 6.70 to 6.90 was only 0.2 of a pH unit, the pCO_2 decrease required to maintain a bicarbonate concentration of 5.3×10^{-3} M was not 50% but approximately two-thirds of 50%. The

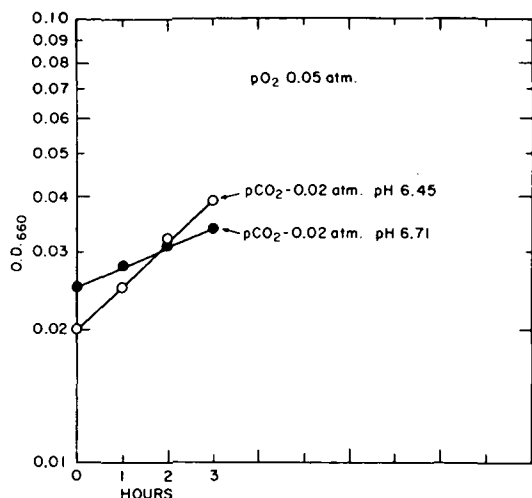


FIG. 3. Same conditions as Fig. 2 except a 4% exponential phase inoculum having an OD_{660} of 0.72 was used.

bottom curve in Fig. 4 illustrates the effect of suboptimal CO_2 (bicarbonate) on growth. A pCO_2 of 0.05 atm which was optimal for growth of stationary phase inocula at pH 6.90 was not effective at pH 6.40 because the pH change decreased the bicarbonate ion concentration to 1.6×10^{-3} M. At the time indicated by the arrow, the pCO_2 was raised to 0.16 atm resulting in an increase in the bicarbonate concentration to 5.3×10^{-3} M (pH simultaneously adjusted to maintain the pH at 6.40). Growth immediately responded by increasing to the expected rate.

DISCUSSION

A discrepancy existed between the O_2 and CO_2 concentrations required for the same initial growth rates of shaken cultures and of gas-sparged cultures. Considerably lower gas concentrations needed for sparged cultures indicated that gas medium equilibration was not achieved in shaken cultures even though a relatively small medium volume was used in a flask with baffles to increase effective surface area (7). These results raise a question about the validity of classifying hydrogenomonads on the basis of oxygen tolerance (*Bergey's Manual*, 7th ed.; 4) determined by various culture methods in different laboratories.

Equilibration of O_2 and CO_2 with the medium can only be assured by direct measure of dissolved gases. This kind of equipment was not available so experimental conditions were adopted which would effectively approach gas medium equilibration in sparged cultures. These included a 30-min gas medium pre-equilibration period, large gas flow rates, high impeller speeds,

a relatively small inoculum, and a short experimental time limited to about two cell doublings. If the rate of gas uptake exceeded the rate of gas diffusion, (i) increased impeller speeds would have affected growth, (ii) the pH would have drifted due to CO_2 depletion as the cell concentration increased, or (iii) the pCO_2 requirement would have increased with time. The most sensitive indicator would have been CO_2 because a small change in the pCO_2 would have had a marked effect on the growth rate. None of these effects was observed. The change in rate of cell growth that did occur after several hours was not associated with a deficiency of one of the gases; it was caused by inhibition by excess CO_2 . Inhibition was relieved by decreasing the pCO_2 to 0.02 atm (pH 6.4).

It was shown in Fig. 2B that the pCO_2 for a maximum rate of growth had narrow limits that were exceeded by plus or minus twofold changes in the CO_2 concentration. This fact permitted a clear establishment of the optimum CO_2 for growth of a postexponential phase inoculum at pH 6.40. The same response was found with exponential phase and with stationary phase inocula, but the absolute CO_2 requirement was different for each type of inoculum. At pH 6.40 to 6.45, exponential phase inocula required a pCO_2 of 0.02 atm (Fig. 3), postexponential phase inocula required 0.05 atm CO_2 , and stationary phase inocula required 0.16 atm CO_2 . The optimum pCO_2 for each inoculum was also related to the pH of the medium; for each 0.3 pH unit increase, a 50% reduction of the pCO_2 was re-

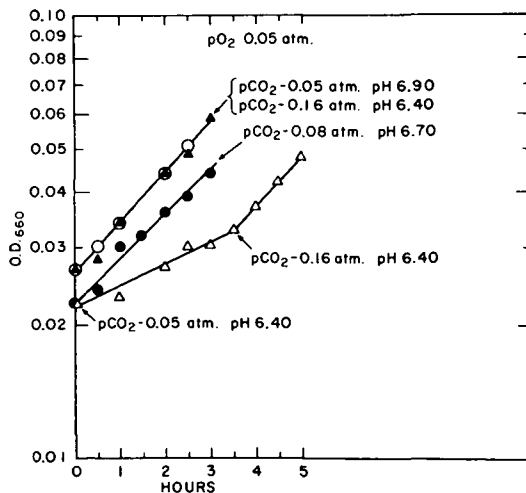


FIG. 4. Same conditions as Fig. 2 except a 1.4% stationary phase inoculum having an OD_{660} of 2.330 was used. In the lowest curve, the pCO_2 was increased to 0.16 atm at 3.5 hr, and the pH was simultaneously adjusted to maintain 6.40.

quired (Figs. 2D and 4). The inverse relationship of a decreasing CO_2 requirement with increasing pH of the medium showed that CO_2 was not the primary substrate. Calculation of the bicarbonate ion concentration in equilibrium with the optimum pCO_2 at each pH tested showed that growth was dependent upon a constant bicarbonate concentration. Immediate exponential growth of inocula from exponential, postexponential, and stationary phase cultures required 6.6×10^{-4} M, 1.6×10^{-3} M, and 5.3×10^{-3} M bicarbonate, respectively. As would be expected, the optimum bicarbonate ion concentrations could be established either by adjusting the pCO_2 at a given pH (Fig. 2 and 4) or, as shown in Fig. 3, by adjusting the pH (from pH 6.71 to 6.45) at a fixed pCO_2 . The various bicarbonate requirements of inocula of different physiological age changed after the culture had grown for one or two generations, and all cultures then needed 6.6×10^{-4} M bicarbonate to maintain exponential growth. This was the same bicarbonate concentration needed initially by exponential phase inocula. Inocula from old cultures thus changed their metabolic requirements during the first hours of exponential growth.

These data show that, in some way, growth of inocula from older cultures can be initiated immediately at maximum specific rates by critical concentrations of bicarbonate. Subcultures of exponentially growing cells also required bicarbonate, implicating the same bicarbonate system. This was further confirmed by the similarity of the sharp optima shown for bicarbonate with all types of inocula tested. If the same bicarbonate system was involved in each case, it appears that this system becomes functionally degenerate during physiological aging. Progressively higher bicarbonate ion concentrations were therefore needed to compensate for the loss of activity. After one or two cell generations, the original activity was restored and a decrease in the bicarbonate concentration was required for continued growth.

Optimal growth conditions of *H. eutropha* (and other cells) generally are those which provide the highest growth rate and the maximum

extent of growth (6, 7). The assumption made is that cells in the inoculum and cells in exponentially growing cultures have the same qualitative and quantitative nutritional requirements. It has been shown that this assumption is applicable only to the special case where the inoculum is from an exponentially growing culture. Since older inocula of *H. eutropha* have a quantitatively different requirement for bicarbonate than is provided in the usual growth medium, a lag period is inevitable.

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